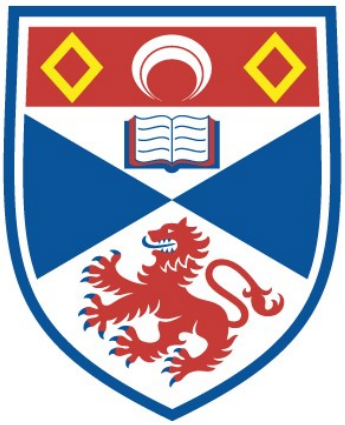
THE RELATIONSHIP BETWEEN DNA DOUBLE-STRAND BREAKS AND MUTATION INDUCTION FOLLOWING TREATMENT WITH X-RAYS AND RESTRICTION ENDONUCLEASES

Baldev Singh

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1992

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Baldev Singh

Thesis submitted for the degree of PhD in Radiation Biophysics
to the Department of Biology and Preclinical Medicine
University of St. Andrews
September 1991

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Declaration

I, Baldev Singh, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any degree or professional qualification

Signed B. Singh Date 30/9/91

I was admitted to the faculty of Science of the University of St. Andrews under Ordinance General No. 12 on 1/10/88 and as a candidate for the degree of Ph.D. on 29/9/89

Signed B. Singh Date 30/9/91

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of PhD.

Signature of the Supervisor Date
Dr. P.E. Bryant

30/9/91

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TABLE OF CONTENTS

LIST OF TABLES, FIGURES AND PLATES

TABLES:

FIGURES:

Chapter 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

PLATES:

ACKOWLEDGEMENTS

I would like to thank my supervisor Dr. Peter E. Bryant for his help, encouragement and patience during the duration of this research project.

I would also like to thank all my fellow colleagues for having provided a pleasant working atmosphere.

A special word of thanks to Mr. John Macintyre for providing the excellent technical support.

I would also like to thank my family for providing the encouragement and support. Thanks to *Kiran*, *Urmi* and *Deepi* for the interest shown. Thanks to my father, who provided the initial financial support which enabled me to come and study for this degree in Scotland.

Finally, I would like to thank British Nuclear Fuels plc for funding this project.

ABBREVIATIONS

tk	Thymidine Kinase
HAT	Hypoxanthine-aminopterin-thymidine
hprt	Hypoxanthine-phosphoribosyltransferase
MEM	Minimum essential medium
FCS	Fetal calf serum
Ara A	9-ß-D-arabinofuranosyladenine
Ara C	arabinofuranosylcytosine
^{3}H-TdR	Tritium labelled thymidine [*Me*-^{3}H] thymidine
TFT	Trifluororothymidine
PBS	Phosphate buffered solution
CHO KI	Chinese hamster ovary cell line
***xrs* 5**	X-ray-sensitive cell line
TK4	*tk*- mutant cell line (induced after 4 Gy X-rays)
L5178Y	Mouse lymphoma cell line
6-TG	6-thioguanine (selection agent to select *hprt*- mutants)
HBSS	Hanks balanced salt solution
RE	Restriction endonucleases
Pvu II	Enzyme purified from *Proteus vulgaris*
Eco RI	Enzyme purified from *Escherichia coli* RY13
Hpa I	Enzyme purified from *Haemophilus parainfluenzae*
Xho I	Enzyme purified from *Xanthomonas campestris*
AluI	Enzyme purified from *Arthrobacter luteus*
AZ	5-azacytadine
EDTA	Ethylenediamine tetra-acetic acid
DNA	Deoxyribonucleic acid
dsb	Double-strand break
ssb	Single-strand break
BSA	Bovine serum albumin
µCi	Micro-Curie [1µCi= 3.7 x 10^4 disintergrations/s]
Bq	Becqueral [equivalent to 1 disintergration/s]
V79	Chinese hamster lung fibroblast cell line
TCA	Trichloroacetic acid
Gy	Unit of radiation
Kv	Kilovolts
BUdR	5-bromodeoxyuridine

IUdR	5-iodo-2'-deoxyuridine
°C	Degree centigrade
CO_2	Carbon dioxide
μM	Micro mol/l = 10^{-6} mol/l
PLD	Potentially lethal damage
EAT	Ehrlich ascites tumour cell line
kbp	Kilo-base pairs
bp	Base pairs
PCR	Polymerase chain reaction
P.E	Plating efficiency
rpm	Revolutions per minute

ABSTRACT

DNA double-strand breaks (dsb) are thought to be major radiation-induced lesions in biological end-points such as cell lethality and chromosome aberrations. Based on the this notion, this project aimed to extended further the investigation of the role of dsb in radiation-induced mutagenesis.

The initial part of the project involved optimising conditions for the mutation assay so as to select for 'true' *tk*- mutants in Chinese hamster cells, following treatment with X-rays. This was important, due to the insufficient previous mutation data involving this locus in Chinese hamster cells. Furthermore, the choice of the *tk* locus over the more commonly used *hprt* locus was based on existing evidence of its higher sensitivity, as found in mutation experiments with the L5178Y mouse lymphoma cell line (Evans et al, 1986).

An initial comparative study was carried out to measure the induced mutation frequency following X-ray irradiation in both the parent Chinese hamster Ovary (CHO KI) cell line and its X-ray-sensitive mutant (*xrs* 5) cell line. This mutant line was chosen because of its characteristic marked deficiency in dsb repair, yet normal ability to rejoin single-strand breaks (Kemp et al, 1984., Costa and Bryant, 1988). This allowed the study of the role of dsb in mutation induction. The enhanced mutation induction observed in *xrs* 5 over that in CHO KI cells suggested the importance of dsb in radiation-induction mutagenesis.

The next experimental strategy adopted involved the use of the DNA synthesis inhibitor, 9-β-D-arabinofuranosyladenine (ara A). The choice of this drug was based on previous work by Bryant and Blöcher (1982) and Iliakis and Bryant (1983) who, using DNA unwinding and neutral velocity sedimentation, showed ara A to strongly inhibit dsb repair. Plateau-phase CHO KI cells were exposed to X-rays alone or in combination with ara A , the latter treatment showing an increased induction of mutations. This suggested the possible existence of dsb which are fixed as mutations in the absence of DNA polymerization, suggesting a sub-class of dsb which may be critical in the steps leading to the induction of a mutation.

The third approach was to use restriction endonucleases (RE) which were introduced into cells by electroporation. This method unlike ionising radiation, induced 'pure' dsb. The use of this method was based on the work of Bryant (1984), who used RE to mimic radiation-induced damage in the induction of chromosomal aberrations. Two different types of RE were used: those which produce blunt- and those which produce cohesive-ended dsb. In all mutation experiments with these enzymes, blunt-ended dsb were found to be more effective in generating mutations compared to cohesive-ended dsb. This suggests a possible further resolution of type(s) of dsb that would be induced by radiation in the ability to induce mutations i.e dependent on the end-structure of the induced dsb. Blunt-ended dsb may thus represent the major type of critical pre-mutational lesions which may be fixed as a mutation, as a result of misrepair. Cohesive-ended dsb may be of lesser importance.

Finally, a RE (Pvu II) which generates blunt-ended dsb was used to induce mutations at the *hprt* locus in Chinese hamster (V79) cells. DNA from mutant cells was analysed using Southern blot and PCR analysis of 3 exons in the *hprt* gene. Some of the mutants (5/15) showed large deletions (representing complete loss of the gene), a change similar to that observed in mutants induced following treatment with ionising radiation (e.g. Thacker, 1986). However, the percentage of large deletion mutants (70%) observed in radiation-induced mutants was higher than that (~34%) obtained with RE-induced mutation data. This preliminary data on the analysis of RE-induced mutations suggests that blunt-ended dsb mimics radiation-induced pre-mutational lesions, resulting in some large genomic changes (e.g. large deletions). However, a larger number of RE-induced mutants would have to be analysed before a more accurate comparison between RE and ionising mutation data can be made.

In summary, this study provides evidence for dsb as a major pre-mutational lesion in cells exposed to ionising radiation, and suggests the existence of a sub-class of dsb in relation to mutation induction. In addition, RE offer the possibility of gaining further understanding of the role of dsb in the origin of mutations such as those caused by deletions.

CHAPTER 1

INTRODUCTION

This thesis describes experiments carried out in an attempt to resolve and establish, out of the broad spectrum of radiation-induced damage, a lesion which may be representative of a pre-mutational lesion. The importance of DNA double-strand breaks (dsb) in relation to a number of biological effects has already been suggested in a number of studies. This accumulated data formed the basis of an investigation into the role of dsb in the process of radiation-induced mutagenesis.

1.1 Mutation research: a brief overview

The field of mutation research gained importance in the early 1900's with some basic questions asked about the nature of mutations, mutation rates and the search for a suitable biological assay which would allow one to select for mutants and thereby measure the induced mutation frequencies following treatment with a number of potential mutagens. This whole field was 're-discovered' following the establishment of Mendels laws of hereditary, by the Dutch botanist Hugo de Vries who coined the word 'mutation' to describe the phenotypic changes observed in the evening primrose (*Oenothera lamarckiana*). This term has since been broadly used to describe a change in the quality, quantity and arrangement of a gene within the DNA of a cell. Mutagenesis describes the above process which may occur spontaneously or may be induced by the action of chemicals or radiation, resulting in a stable heritable change in the progeny of an individual cell. Some mutagenic changes produce deleterious lethal effects while others may remain 'silent' and may not be manifested for many generations.

Due to the long term effects associated with mutations, the nature and mechanisms involving the induction of mutations have always been the subject of active research. This is evident from some of the early proposals put forward for the induction of mutations e.g. Bateson (1928) put forward the presence-absence theory according to which all mutations were due to loss of normal genes. This theory was, however, contradicted by the observation of reverse mutations. Other investigators suggested that mutations arise as a result of 'errors' in gene replication, this notion being based on the hereditary changes observed in subsequent generations.

The two most important findings for advancement in the field of mutation research have been the unambiguous demonstration of the mutagenic properties of X-rays (Müller, 1927), and the elucidation of the 3-dimensional helical double-stranded structure of the DNA (Watson and Crick, 1953). The discovery of the mutagenic effects of X-rays provided for the first time an experimental probe into the methods of altering the gene structure and following the subsequent changes. In addition, a new class of mutations could be isolated at high frequencies thus allowing efficient detection of mutations using various assays. The base complimentary structure of the DNA helix accounted for the fidelity of gene replication and allowed the induction of mutations to be explained on the basis of nucleic acid chemistry. The above information led to investigations into the cellular mechanisms associated with mutations such as repair and expression of damage which currently play a central role in a further understanding of mutation induction.

1.2 Basic types of mutational changes

Mammalian cells are equipped with a set of enzymes which are responsible for maintaining the integrity of the genetic code by repairing damage induced in the DNA, either spontaneously or by both physical and chemical agents. Most types of mutational (genetic) changes generally observed can be classified into one of three categories which include: (a) changes at the gene level (b) structural chromosomal changes (c) changes in the number of chromosomes.

Two of the earliest types of mutations recognised at gene level were base substitutions and frameshift mutations. Frameshifts had been shown to be due to the addition or deletion of base pair(s) which subsequently shifted the triplet reading frame so that the coding sequence is altered. Base pair substitutions had been recognised much earlier (Watson and Crick, 1953) and were suggested to result from a tautomeric shift in the base which might lead to misrepair during DNA replication. One of the most common types of base substitution observed in irradiated cells is the G.C to A.T transistion (Tindall et al, 1988).

The second category arises as a result of primary damage to an individual chromosome which involves the breakage of the chromatids. This break may restitute in the original form as a result of a repair process, and in this case no cytological damage is observed. If the break remains unrejoined, this may result in the death of the cells after passing through one or two phases of mitosis. This type of damage (when it occurs in germ cells) often results in the death of the embryo at an early stage, hence constituting what is termed as a 'dominant lethal' mutation. Alternatively, the breaks may rejoin in a different order from the original giving rise to chromosome rearrangements. Types of mutational change in this category include translocations, inversions and deletions. Some of these changes e.g. deletions, may uncover the deleterious effects of a pre-existing recessive gene. This has an added importance since deletion mutations have been shown to represent a major class in radiation-induced mutagenesis (Vrieling et al, 1985., Thacker et al, 1990).

The third type of genetic change involves a change in the number of chromosomes, termed as aneuploidy (abnormal number of chromosomes). This can arise when homologous chromosomes do not separate during cell division so that both chromosomes move into the same daughter cell. This is termed as non-disjunction. Aneuploidy may also occur via endoreduplication which may give rise to a tetraploid cell followed by a chromosome loss. These types of alterations have been associated with a number of human diseases such as Downs syndrome. A better understanding of the molecular mechanism for mutation induction will allow a better understanding of some of the genetic diseases associated with these mutational changes.

The complex structure and organisation of mammalian chromatin has limited our understanding of the process involved in the induction of a mutation. Nevertheless, some of the basic steps have been elucidated, the salient features being summarised in figure 1.1.

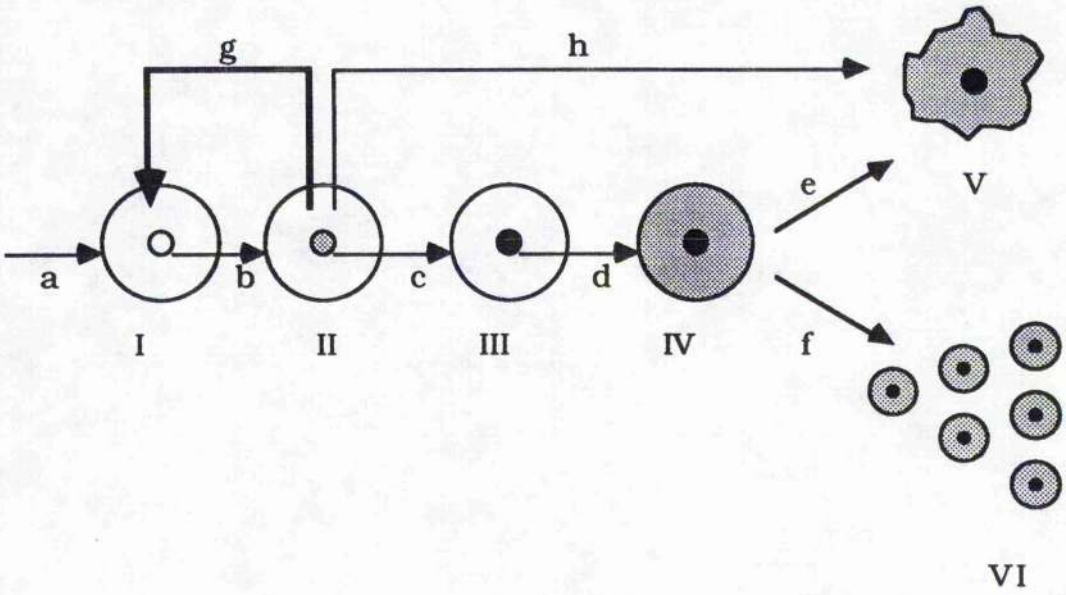


Figure 1.1. Mutagenic pathways for the induction of a mutation. I. Non-mutated gene in wild-type cell line. II. Gene with a pre-mutational lesion. III. Mutated gene in non-mutated cell. IV. Mutated gene in mutant cell. V. Dead mutant cell. VI. Mutant cells further divide. The pathways include (a) Exposure of cells to the potential mutagen (b) Production of a premutational lesion (c) Fixation of premutational lesion (d) Expression of non-lethal damage resulting in the production of a mutant cell (e) Death of mutant cell due to a lethal mutation (f) Formation of mutant cell clone (g) Repair of pre-mutational lesion, restoring the normal gene (h) Unrepaired damage resulting in cell death.

1.3 Ionising radiation-induced lesions

In order to understand and resolve the type(s) of initial damage which, if fixed, may be responsible for the induction of a mutation, it is important to know the types of lesions induced in the DNA of mammalian cells following exposure to ionising radiation. There are generally two modes of action by which ionising radiation is thought to induce damage in the DNA which is accepted to be the main sensitive target for radiation damage. These are classified as direct and indirect modes of damage. The former occurs as a result of high local energy deposition directly within the DNA molecule (Goodhead and Brenner,

1983) while the latter includes damage mediated through oxygen derived free radicals, particularly hydroxyl radicals which may attack the DNA molecule (Teoule and Cadet, 1978., Hutchinson, 1985).

The major types of damage associated with both the above modes of action include base damage, DNA strand breaks and DNA-DNA and DNA-protein cross-links (Ward, 1985., Teoule, 1987). The importance of base damage in radiation-induced mutagenesis has been well established in bacteria, bacteriophages and lower eukaryotes (Glickman et al, 1980., Levin et al, 1982., Conkling et al, 1976., Malling and de Serres, 1973). The most common type of radiation-induced base damage includes transistional and transversional changes between A-T and G-C base pairs (Glickman et al, 1980). However, the role of base damage in radiation-induced mutagenesis in mammalian cells remains unclear. This notion is supported by the observation that ionising radiation does not increase the frequency of mutants at the Na^+/K^+-ATPase locus which has been shown to tolerate point mutations only (Arlett et al, 1975., Thacker et al, 1978., Liber et al, 1983). However, in the same experiments, the frequency of mutations at the *hprt* locus are significantly increased (Arlett et al, 1975., Thacker and Cox, 1975., Liber et al, 1983).

There is increasing evidence which points to DNA strand breaks as important primary radiation-induced lesions responsible for causing cell inactivation and mutations (Cole et al, 1980). DNA strand breaks can be sub-divided into two categories namely single-strand breaks (ssb) and double-strand breaks (dsb). Both these represent breaks in the covalently bonded sugar-phosphate chains of the DNA helix. Ssb are thought to be induced at a higher frequency (~1100/Gy/genome) in comparison to the dsb (~40/Gy/genome) (Blöcher, 1982., Van der Schans et al, 1982).

The other types of damage include the DNA-DNA and DNA-protein cross-links which are induced at a low frequency compared to ssb (133/Gy/genome) and seem to play a relatively minor role in contributing to the effects observed in cells exposed to ionising radiation.

Repair of damage in the DNA is important to maintain the integrity of the genetic code. A complex enzymatic mechanism exists in cells to ensure that DNA is replicated with a high fidelity, due to the mismatch repair activity to remove rare mis-incorporated base

residues, which may have escaped the cell's proof-reading ability during DNA replication. The presence of such a highly efficient repair system in mammalian cells is supported by the view that an absorbed dose of 1 Gy leads to an average of less than 1 'lethal' lesion in normal cells.

Both base damage and ssb are thought to be repaired efficiently by excision repair with a high degree of fidelity (Painter and Young, 1972., Fox and Fox, 1973) hence these are postulated not to be lesions responsible for cell death or mutations in irradiated cells. Dsb have been suggested to represent critical lesions which are responsible for some of the cellular and cytogenetic effects observed in irradiated cells (Hutchinson, 1974., Frankenberg et al, 1984., Blöcher and Pohlit, 1982., Van der Scans et al, 1982., Radford, 1986., Ho, 1975., Resnick and Martin, 1976). The importance of dsb is supported by experiments carried out with the rad 52 yeast mutant strain (deficient in dsb repair) in which 1-2 dsb are found to constitute a lethal event (Ho, 1975., Resnick and Martin, 1976., Frankenberg et al, 1984). This view is further supported by the increased radiosensitivity observed in mutant cells which are known to have a reduced level of dsb repair (Kemp et al, 1984., Evans et al, 1987., Wlodek and Hittlemann, 1987., Costa and Bryant, 1988., Zdzienicka et al, 1988).

One of the complications associated with trying to link a specific ionising radiation-induced lesion to an specific end-point is due to the large number of lesions generated (figure 1.2).

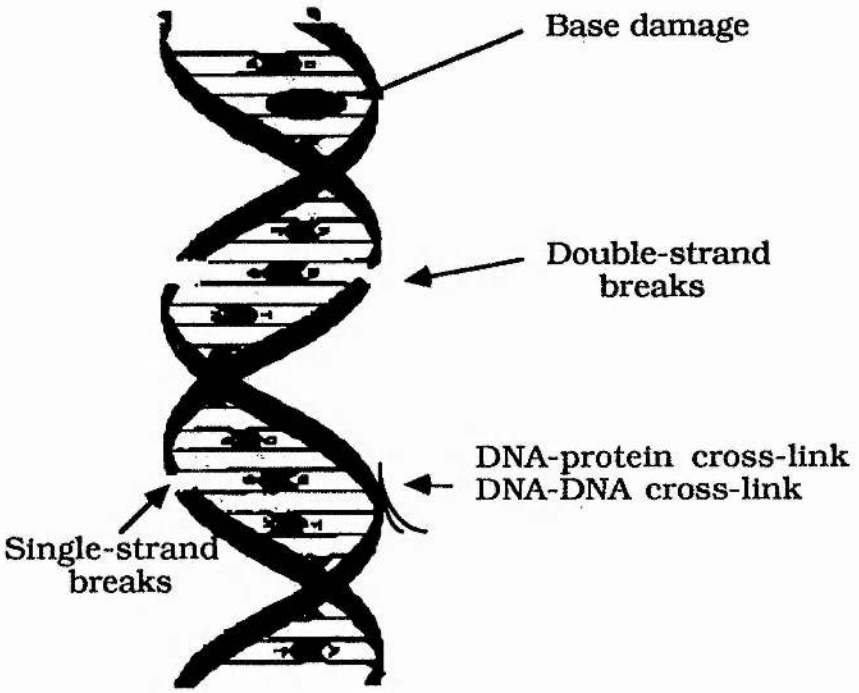


Figure 1.2. Primary lesions induced in DNA following treatment with ionising radiation.

This has been overcome more recently with the use of restriction enzymes (RE) to generate dsb, hence mimic radiation-induced damage (Bryant, 1984). Such studies have provided a strong correlation between dsb and induction of aberrations (Bryant, 1984., Obe et al, 1985), cell lethality (Bryant, 1985), mutation induction (Obe et al, 1986) and oncogenic transformation (Bryant and Riches, 1990). These studies emphasize the usefulness of RE as an important tool for further understanding of radiation-induced damage. However one should be cautious in comparing RE data to the radiation data. RE-induced dsb generate 'clean' breaks with a 3'-hydroxl and 5'-phosporyl termini whereas radiation-induced lesions are more likely to have 'dirty' ends hence both types of lesions will be handled by a different set of repair enzymes (Bryant, 1988., Ward, 1986., Ward et al, 1987).

1.4 Correlation of DNA double-strand breaks with other cytogenetic endpoints

Dsb have been suggested to play an important role in the formation of chromosomal aberrations (Bender et al, 1974., Natarajan et al, 1980). This is thought to be as a result of non-repair or misrepair of dsb giving rise to deletions and exchange types of chromosome aberrations (Bender et al, 1974). The importance of dsb in the induction of chromosome aberrations is supported by Natarajan and Obe (1978) who showed that when additional dsb were introduced into X-irradiated mammalian cells via conversion of ssb into dsb by *Neurospora* single strand endonuclease, a corresponding increase in the frequency of chromosome aberrations was observed.

Additional evidence was provided with the introduction into cells of RE that induced only dsb. These showed an increased production of chromosome aberrations, providing more direct evidence for the involvement of dsb in the formation of aberrations (Bryant, 1984., Natarajan and Obe, 1984). Inhibition of dsb repair has also been shown to result in an increased fixation and subsequent formation of chromosome aberrations (Iliakis et al, 1988., Mozdarani and Bryant, 1987, 1989). These experiments were based on earlier observations in which an increased frequency of chromosome aberrations was observed in mutant cells which are known be defective in dsb repair (Kemp and Jeggo, 1986., Darroudi and Natarajan, 1987a). Cells irradiated in the presence of ara A, an inhibitor of dsb repair (Bryant and Blöcher, 1982) also showed an increased formation of CA (exchanges) This was suggested as evidence for the misrepair of dsb (Mozdarani and Bryant, 1987).

1.5 Repair of double-strand breaks

The repair of dsb is a controversial subject due to the conflicting repair data obtained while working with different biochemical systems. Results seem to depend on the type of assay used. Some of the methods used to measure dsb repair include the neutral velocity sedimentation technique (Lehmann and Stevens, 1977., Blöcher, 1982), DNA unwinding technique (Bryant and Blöcher, 1980), DNA

precipitation assay (Olive, 1988), neutral filter elution (Bradley and Cohen, 1979) and the more recent methods of pulse-field gel electrophoresis (e.g. CHEF: clamped homogenous electrical field, Blöcher et al, 1989). In order to investigate the mechanism of dsb repair, a number of bacterial mutant cells which are known to be defective in a specific repair pathway have been used. Results from these experiments have suggested the requirement of an intact, undamaged region of a homologous DNA molecule as a template during repair synthesis (Krasin and Hutchinson, 1977., Weibezahn and Coquerelle, 1981., Picksley et al, 1984., Lopez and Coppey, 1987). This evidence supports models which propose that dsb repair requires a recombination-type mechanism (Resnick, 1976., Szostak et al, 1983). The involvement of recombinational repair is consistent with the observations that dsb and small deletions stimulate recombination (Szostak et al, 1983). Recombinational repair in bacterial cells is thought to involve exonucleases which degrade the ends of the induced dsb, followed by resynthesis of the intervening gap using the DNA sequence from the homologous chromosome. This process thus requires the activity of DNA polymerase during the resynthesis of the strand, in addition to DNA ligase activity to complete the final rejoining of the ends. The importance of the above type of repair is supported by the observation of increased radiosensitivity of the rad 52 mutant of yeast known to deficient in repair of dsb and which is also found to have a defective recombination repair process (Resnick, 1975).

In contrast to the extensive literature on dsb in prokaryotic cells, dsb repair processes in mammalian cells are not well understood. The radiosensitive mutant *xrs* 5 cell line which is known to be defective in dsb repair (Kemp et al, 1984) has been found to have a reduced ability to carry out recombination in an *in vitro* assay. Hence, recombination repair has been suggested as a possible mechanism by which dsb are repaired using the homologous DNA to ensure accuracy of rejoining in mammalian cells (Debenham et al, 1987). However, based on the fast repair component observed in dsb repair, Weibezahn and Coquerelle (1981) suggested this as evidence for a simple ligation mechanism for dsb repair. Radiation is thought mostly to generate breaks with 'dirty' ends, with a 3'-hydroxyl and 5'-phosphoryl which must be trimmed by an exonuclease prior to any

ligation step (Lennartz et al, 1975). This argues against the view that a direct ligation repair process exists in mammalian cells.

Fidelity of dsb repair has been suggested to be one of the determining factor which accounts for the increased lethality observed in dsb repair-defective mutant cells. Evidence for this is provided by experiments done with cells from individuals with the autosomal recessive genetic disease, ataxia telangiectasia (AT). AT cells have not been found to be deficient in the repair of bulk dsb (Lehmann and Stevens, 1977). The increased radiosensitivity is attributed to inaccuracies in dsb rejoining rather than lack of repair (Cox et al, 1984, 1986., Debenham et al, 1987). The low fidelity in AT cells is suggested to account for the relatively large genomic deletions and rearrangements at sites of dsb. Cox et al (1986) postulated the existence of a competition between ligation and exonuclease digestion of dsb with a shift towards the latter step in AT cells.

1.6 Assays currently used to detect mutation

Most of the *in vitro* mammalian cell mutation systems are used to measure forward mutational changes. The advantage of working with such systems is that they offer a large number of restriction sites available for mutational changes within the target locus, provided the gene(s) code for a product which is not essential for the survival of the cell. Different mutation assays measure different types of mutations with varying efficiencies which has stimulated interest in the development of a variety of mutation assays. Despite the large number of laboratories which may use a particular assay, the establishment of optimum selective conditions under which all types of mutants can be detected is a complex task which may often be responsible for the contradictory mutation data between different laboratories.

Some of the more commonly used mutation systems for the detection of forward mutations *in vitro* are briefly discussed.

1.6.1 *Hypoxanthine-guanine Phoshoribosyltransferase Locus (hprt)*

The X-linked *hprt* locus is the most extensively used locus for mutation studies in cultured mammalian cells (Albertini and DeMars, 1973., Van Zeeland and Simons, 1976., Thacker et al, 1976, 1977., Shaw and Hsie, 1978). In cell cultures, mutations in the *hprt* locus can be selected for by growing cells in 6-thioguanine containing medium (selection medium). The types of mutational changes in the *hprt* locus following treatment with ionising radiation have been well characterised in Chinese hamster V79 cells (Vrieling et al, 1985., Thacker, 1986., Fuscoe et al, 1986), Chinese hamster ovary cells (Breimer et al, 1988., Stankowski and Hsie, 1986., Gibbs et al, 1987) and in human lymphocytes (Liber et al, 1987). Molecular analysis of mutants induced following treatment with ionising radiation suggested that about 70% have undergone gross structural damage to the DNA resulting in deletions of large sections of the sequence (Kavathas et al, 1980., Orr et al, 1982., Graf and Chasin, 1982., Thacker, 1986., Yandell et al, 1986., Liber et al, 1987). In cases of large deletions, the extent of these has been approximately determined by Southern analysis in the *hprt* gene (Thacker et al, 1990). All this extensive molecular analysis at this locus has been made possible due to the cloning of the complementary DNA (cDNA) sequence for the *hprt* gene (Konecki et al, 1982).

The mouse *hprt* gene is divided into 9 exons which vary in length ranging from 18 to 593 base pairs This allows one to further localise and map the type of changes (deletions) within different parts of the gene, i.e either at the 3' or 5' end. This type of analysis is based on the availability of sequences flanking the coding regions for the exons (primers), thus allowing the polymerase chain reaction (PCR) to amplify specific regions of the gene (exons) to determine the extent and position of damage (Rossiter et al, 1991). A close homology has been found to exist between the HPRT protein sequences of mouse, hamster and humans (Konecki et al, 1982), which is advantageous for interspecies extrapolation of the mutation data. However, mutagenic mechanisms such as homologous mitotic recombination or gene conversion are not available at such a locus due to its X-linked nature (single copy). Some of the additional variables which are suggested to determine the sensitivity of a locus include its chromosomal

localization, ploidy and linkage to essential genes (Kronenberg and Little, 1989); hence large scale deletion at this locus may be lethal therefore going undetected due to its hemizygous nature.

The *hprt* gene is extensively used since its only single copy avoids confusion when analysing types of mutations at a molecular level (due to non-specific binding). The only non-specific binding is to one or more functional genes (pseudogenes) which hybridize to the probes used (Fuscoe et al, 1983). The limit of resolution for detecting mutational changes of this locus is approximately 500 bp.

1.6.2 *Adenine Phosphoribosyltransferase Locus (aprt)*

This locus, coding for the non-essential enzyme adenine phosphoribosyltransferase, is autosomally located in mammalian cells (Tischfield and Ruddle, 1974., Kozak et al, 1975). It offers an attractive choice of target locus for mutation studies due to its relatively small size (4 Kb). This reduced size, in addition to the large number of available restriction sites within the *aprt* gene, allows a better resolution of the extent of mutational damage (Meuth and Arrand, 1982., Nalbantoglu et al, 1983). Base changes, insertions and deletions as small as 25 bp have been detected at this locus (Nalbantoglu et al, 1983). Although autosomally located, some cell strains with a single copy of the gene have been identified which are successfully used in mutation studies (Chasin, 1974., Thompson et al, 1982). *Aprt-* mutants can be selected in single-step using resistance to the toxic analogue, 8-aza-adenine.

1.6.3 *Dihydrofolate Reductase Locus (dhfr)*

Mutant cells at this locus are isolated by resistance to methotrexate (MTX), a 4-amino analogue of folic acid. A mutation in the *dhfr* locus reduces the cells affinity for MTX. The autosomal nature of this locus interferes with analysis of molecular changes. However, this can be overcome by using cell strains which have been found to be hemizygous for this locus (Uraluab et al, 1980). A large number of deletions and rearrangements have been identified at this locus

following treatment with ionising radiation (Uralaub et al, 1980). The resolution of this locus is approximately 100 bp. Flintoff et al (1976) have demonstrated resistance to MTX occuring as result of permeability changes in the cell membrane, thus excluding the entry of the analogue into cells. Due to the existence of such a possible non-genetic change, much more work has yet to be done in optimising experimental conditions for the selection of mutants at this locus before it is accepted as a choice of mutation assay.

1.6.4 *Na^+/K^+-ATPase Locus*

This locus codes for the membrane-bound enzyme Na^+/K^+-activated ATPase, which is vital for the functioning of the Na^+/K^+ 'pump' which maintains the control of these ions across the cell membrane. Mutations at this locus can be selected by resistance to Ouabain, a steroid which in normal cells binds to the enzyme causing an ionic imbalance resulting in cell death (Baker et al, 1974). The types of mutational changes which can be isolated at this locus are more restricted in comparison to those observed at the other loci e.g. *hprt*. Evidence suggests that only small changes, probably point mutations, can be tolerated at this locus without being lethal. This is demonstrated by the loss of resistance to Ouabain while inducing *hprt*$^-$ mutants in cells exposed to ionising radiation, supporting the view that large deletions are lethal. The advantages of this locus are its relative stability and the fact that it is well characterised. However, there are also some disadvantages, including a relatively low mutant frequency, the existence of some cells for which Na^+/K^+-ATPase activity is required for survival and as mentioned above its ability to detect only single-base changes.

1.7 Thymidine kinase mutation assay

The locus coding for the enzyme thymidine kinase (TK; EC 2.7.2.21) is known to be autosomally located in mammalian species e.g. on chromosome 17 in man (Miller et al, 1971), chromosome 11 in mouse (Kozak and Ruddle, 1977) and on chromosome 7 in Chinese

hamster cells (Stallings and Siciliano, 1981). Thymidine kinase catalyses the phosphorylation of pre-existing thymidine to thymidine 5'-monophosphate, which is subsequently used for DNA synthesis. There are two forms of the enzyme, one localized in the mitochondria and the other in the cytosol. The cytosolic enzyme plays the major role during DNA synthesis (peak activity during S phase of the cell cycle) while the mitochondrial enzyme activity remains constant during the cell cycle.

The presence of thymidine kinase is not essential for the survival of a cell. The *de novo* biosynthesis of thymidine monophosphate by the enzyme thymidylate synthetase constitutes the normal pathway by which cells meet their thymidine requirements. The thymidine kinase catalysed reaction represents one of the several 'salvage' pathways for the cell (Kit et al, 1963) hence the activity of thymidine kinase can vary substantially without affecting cell survival (Breslow and Goldsby, 1969), an important aspect for any mutation assay. Due to the autosomal nature of the *tk* locus, two copies of this locus will normally be present in the parent wild-type cell strains. This makes isolation of *tk*- mutant cells extremely difficult in cultured mammalian cells (Clive et al, 1972., Roufa et al, 1973). However, there is increasing evidence that some lines occur naturally or during the evolution of the line to give rise to cells which are hemizygous/heterozygous (one effective functional gene) for the *tk* gene. These can be successfully used for mutation studies (Clive et al, 1972., Fox, 1974). The mouse lymphoma L5178Y $tk^{+/-}$ 3.7.2 C cell line has been widely used in studies to quantify the potential of chemical and physical agents to induce mutations at the autosomal heterozygous *tk* locus (Clive et al, 1979., Moore et al, 1985 a,b). Yandell et al (1986) used a human B-lymphoblastoid cell line for detection of mutations at the *tk* locus. This line is heterozygous for the *tk* locus, with one functional and one-non-functional allele which is thought to be due to a single base change in one of the alleles. Similarly, Evans et al (1986) successfully used the mouse L5178Y strain LY-S and its parental strain LY-R to make comparative mutation studies at the *tk* locus.

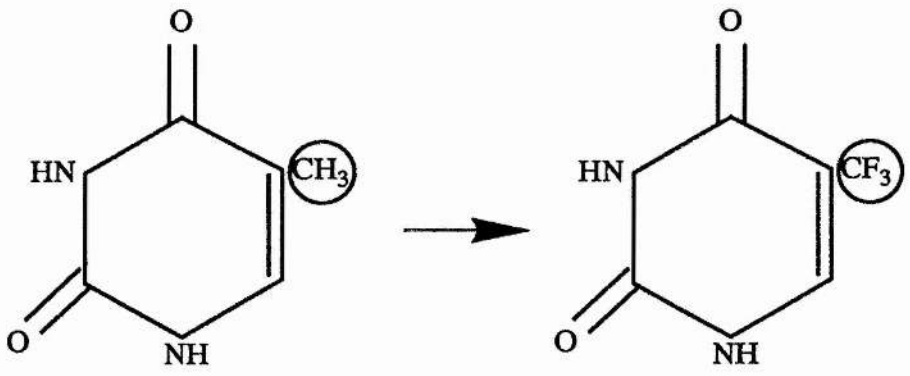
In some preliminary studies carried out in our laboratory, the *tk* locus in Chinese hamster ovary (CHO KI) cell line was found to be an efficient locus to measure mutations following treatment with X-rays.

This involved a single-step selection system. Mutants arose with a high frequency following treatment with ionising radiation. Based on these preliminary results, this mutation assay was further refined and used for the experiments described in this thesis. Furthermore, the choice of the *tk* locus rather than the hemizygous *hprt* locus is to some extent the result of increasing evidence indicating the higher sensitivity of the *tk* locus to ionising radiation (Clive et al, 1980., Yandell et al, 1986., Stankowski and Hsie, 1986., Evans et al, 1986., DeMarini et al, 1989). This difference in the two loci (*tk* and *hprt*) has been suggested as evidence for a class or classes of mutations which may be recovered at heterozygous autosomal loci that are not recovered at the X-linked hemizygous locus. Using the mouse lymphoma L5178Y assay, two different types or classes of mutations have been thought to exist at the *tk* locus. This view is based on the existence of large and small mutant colony sizes. On the basis of this observation, it has been suggested that the small colonies may arise from the induction of mutagenic chromosome damage, while the large colonies are representative of gene mutations (Clive et al, 1979., Turner et al, 1984). The notion that the majority of small colony (mutant) cells have chromosome abnormalities is supported by the increased frequency of chromosomal aberrations (i.e. breaks and rearrangements). Mutants which recovered following the exposure to ionising radiation have frequently been shown to be the result of multilocus lesions (Wolff, 1971., Evans et al, 1986). This will affect flanking genes required for cell survival and growth. This would result in loss of mutant cells recovered at the *hprt* locus while, presumably, the genes for survival and growth on the homologous chromosome remain intact at the heterozygous loci (*tk*), thus furnishing the critical gene product (for survival and growth).

1.7.1 *Use of TFT as a selection drug*

Flourinated pyrimidines have been shown to inhibit the growth of transplanted tumours in rodents and in patients suffering with advanced cancers of the breast and gastro-intestinal tract (Heidelberger et al, 1958). This was found to be due to the phosphorylated form of the flourinated pyrimidine which is a powerful inhibitor of thymidylate synthetase (Heidelberger, 1965). In addition

to this, the pyrimidine analogues are incorporated into the DNA blocking replication. Based on these observations, the use of flourinated analogues of normal nucleotides has since been extended to the isolation of *tk*- mutant. The main mutation assay described in this thesis makes use of a toxic nucleotide analogue of thymidine, 5-trifluoromethyl-2'-deoxyuridine commonly known as trifluorothymidine (Gottschling and Hiedelberger, 1963). The structure of TFT is similar to that of the thymine except for substitution with fluorine at the 5' position (figure 1.3). A similar substitution can be made with bromine and iodine giving rise to 5-bromo-2'-deoxyuridine (BUdR) and 5-iodo-2'-deoxyuridine (IUdR) respectively.



Thymine Trifluorothymine (TFT)

Figure 1.3. Chemical structure of trifluorothymidine

Both BUdR and IUdR have also been used to select for tk-mutants (Clive et al, 1972., Fox, 1971., Roufa et al, 1973) on the basis that they induce a considerable inhibition of cell division and slow killing of the non-mutagenised cell population (Fujiwara et al, 1970). However, TFT was used as the selection drug based on existing evidence for its rapid inhibition of cell division (Adair and Carver, 1979) and its non-mutagenic property in mammalian cells unlike BUdR and IUdR (Huberman and Heidelberger, 1972). Since the size of the trifuoromethyl group (2.44 Å) in TFT is nearly the same as that of the methyl group in the normal thymine base (2.0 Å), it is most likely

that the phosphorylated form of TFT is incorporated into the DNA (Gottschling and Hiedelberger, 1963), thus interfering with the process of replication in normal cells but allowing survival of the *tk*- mutant clones. The mode by which TFT selects for presumptive *tk*- mutants is summarized in figure 1.4.

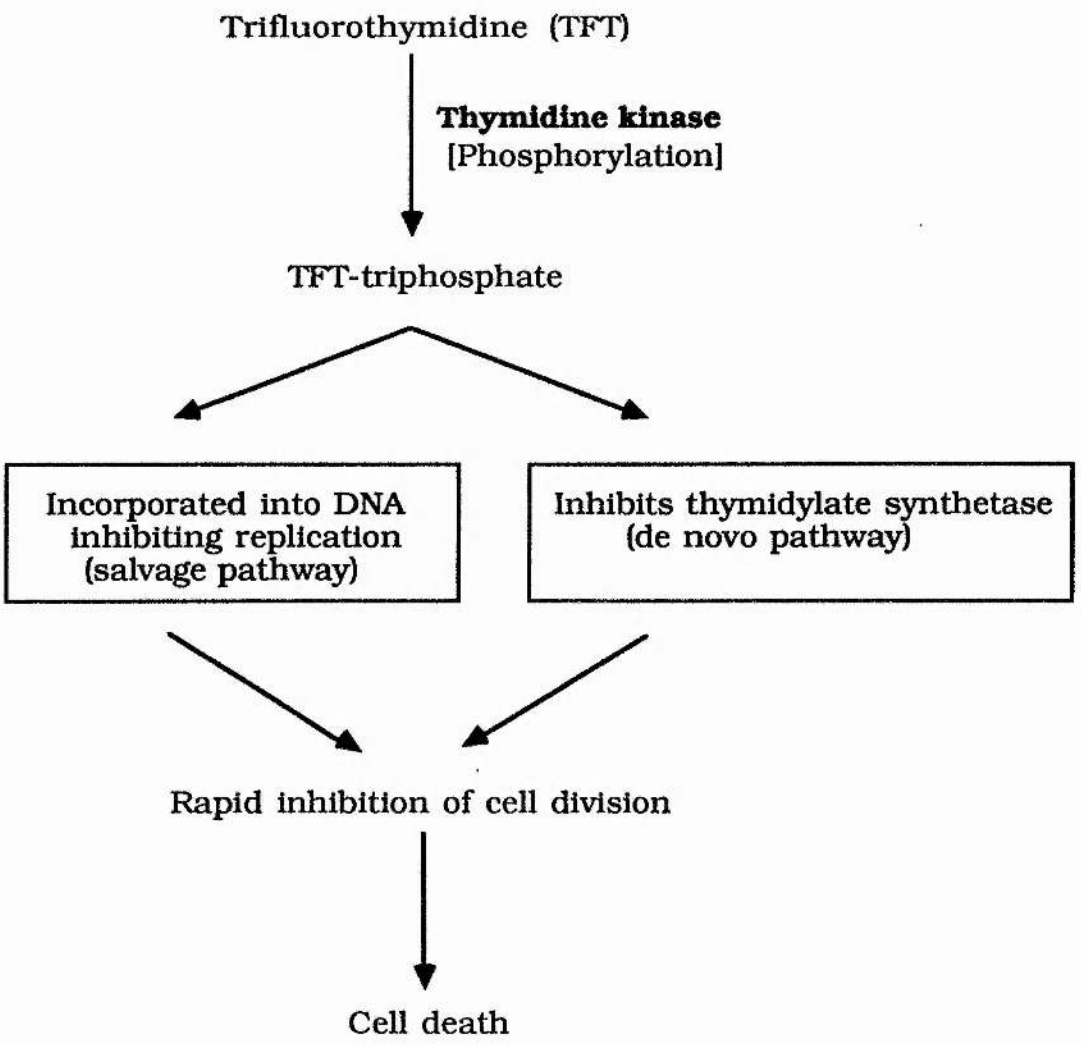


Figure 1.4. Summary of the steps involved during the plating of cells in the selection medium (TFT).

1.8 Specific aims of this project

From all accumulating data discussed above, it is evident that radiation-induced dsb play an important role in the observed cellular and cytogenetic effects in cells exposed to ionising radiation. In light of this, the main aim of this project was to investigate into a possible relationship between dsb and the process of mutation induction hence providing evidence for dsb as important pre-mutational lesion.

The strategy was first to establish a mutation assay and conditions necessary to successfully isolate 'true' mutants (*tk*-) following treatment with various types of treatments. The autosomal locus coding for the enzyme thymidine kinase in Chinese hamster cells was used as the target locus for the most part of the studies described in this thesis. This locus was used due to its increased sensitivity in comparison to the *hprt* locus which might be due to its ability to detect certain classes of mutations which are recovered at *hprt* locus.

The first approach was to compare the induction of mutations in the parent Chinese hamster ovary (CHO KI) wild-type cell line and in its X-ray sensitive mutant strain, *xrs* 5. This mutant line was chosen on its characteristic marked deficiency in dsb repair, yet showing a normal repair of ssb (Kemp et al, 1984), thus allowing one to follow the role of a reduced dsb repair on mutation induction.

The second strategy was to use a DNA synthesis inhibitor which is also known to inhibit the repair of dsb. Both the CHO KI and *xrs* 5 cell lines were exposed to X-rays alone or in combination with the dsb-repair inhibitor followed by measurement of the relative frequency of mutation induction. In this series of experiments, the effect of dsb-repair inhibition on the repair of damage was also measured at a cytogenetic level (aberrations).

The third approach was to use restriction endonucleases (RE) which are known to generate dsb in an attempt to simulate radiation-induced dsb (Bryant, 1984). This allowed one to study the role of 'pure' dsb on mutation induction unlike in case of radiation-induced damage which generate the broad spectrum of damage. In addition to this, different RE which generate different types of dsb were used to further distinguish between the type of dsb (blunt- or cohesive-ended dsb) most critical in the steps leading to the induction of a mutation.

During these experiments, RE were introduced into the cells using a cell electroporation technique which was found to be a much more effective and reliable method than previously used methods of permeabilization (e.g. inactivated Sendai virus).

The fourth aim of this project was to investigate into the type(s) of molecular changes associated with dsb-induced mutational changes. In this preliminary study, a RE was used to induce mutations at the *hprt* locus in Chinese hamster (V79) cell line. The mutation changes at the *hprt* locus were analysed using Southern bloting and PCR analysis. RE-induced mutation profiles was compared with that observed in radiation-induced mutants from previous work.

Each of the above experiments is described in individual chapters with a introduction at the beginning of each chapter followed by a discussion at the end.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Introduction

This chapter describes the general material and methods which were routinely used during the course of this project. This includes cells, culture conditions and the basic mutation assay used to isolate mutations at the autosomally located *tk* locus in hamster cells. The basic mutation assay used has been described in general terms, however any additional modifications in any part of the assay e.g the exposure of cells to the potential mutagen will be described in the materials and method section of the respective chapter.

2.2 Cells, culture conditions and materials

During the course of this project, all cell lines used, Chinese hamster ovary (CHO KI), X-ray-sensitive mutant cell line (*xrs* 5), Chinese hamster (V79) cell line were routinely grown as monolayers in 75 cm^2 tissue culture grade plastic flasks (Sterlin) and maintained in exponential growth in 10 ml Eagles Minimum Essential Medium (MEM) supplemented with non-essential amino acids and 10% (v/v) fetal calf serum (FCS). Flasks were gassed with 5% CO_2 and incubated at 37°C. Cells were passaged twice a week to maintain cells in exponential growth in all experiments unless otherwise mentioned. For passaging, medium was removed and cells detached from the surface by trypsinization using a trypsin/EDTA solution. Cells were rinsed twice with 3 ml of trypsin/EDTA solution and incubated for 6 minutes at 37°C. After this period, cells were found to be detached from the surface of flask (seen as rounded free floating cells when viewed under an inverted microscope). To each flask, 5 ml of MEM/FCS was added and the suspension pipetted two to three times to give rise to a single cell suspension. To determine the cell concentration, 100µl of suspension was mixed with 9.9 ml of isoton solution and counted in a coulter counter (model D) using the following settings: Threshold=20; Attenuation=8; Aperture current= 0.017. The composition of routinely used solutions is described below:

(a) *Eagles minimum essential medium (MEM)*

100 mls Minimum essential medium (x10 concentrate Gibco)
10 mls Penicillin + Streptomycin,
10 mls Glutamine
10 mls Non-essential medium (Gibco)
30 mls Sodium bicarbonate
840 mls double-distilled water

(b) *Trypsin/EDTA*

Trypsin (Difco) 0.05 % with 0.7 mM EDTA (BDH) in Phosphate Buffered Saline.

(c) *Trifluorothymidine supplemented medium (TFT/MEM)*

Crystalline trifluorothymidine (trifuorothymine deoxyriboside, Sigma) was weighed and dissolved in distilled water followed by filtration through a millipore filter (0.22 μM, Flow laboratories). To give the final working concentration of 3μg/ml, the stock was diluted in MEM/FCS. All TFT supplemented solutions were protected from flourescent light.

(d) *HAT-supplemented medium (HAT/MEM)*

HAT media supplement (50x concentrate, Flow Lab.) was diluted in MEM/FCS to give 1x concentrate solution with the following composition: hypoxanthine (100μM), aminopterin (0.4μM) and thymidine (16 μM).

(e) *6-thioguanine supplemented medium*

A stock solution of 6-thioguanine (2-amino-6-mercaptopurine, Sigma) was prepared by dissolving in HBSS followed by millipore filtration (Flow Lab.). The final working concentration of 1μg/ml was obtained by subsequently dissolving in MEM/FCS.

(f) *Hank's Balanced Salt Solution (HBSS).*

0.14M NaCl (8.0g/l), 5.4mM KCl (0.4g/l), 0.34mM $Na_2HPO_4.2H_20$ (0.06g/l), 0.44mM KH_2PO_4 (0.06g/l), 6mM $MgSO_4.7H_20$ (1.5g/l), 5.6mM D-Glucose (1g/l), 4.2mM $NaHCO_3$ (0.35g/l). (All BDH).

(g) *Hank's Balanced Salt Solution (modified) + BSA.*

HBSS (see above) + 1% (10g/l) Bovine Serum Albumin (Fraction V). (BDH). (designated - HBSS/BSA)

(i) *Fetal calf serum (FCS)*

For the growth of cell cultures and mutageneisis experiments, normal medium (MEM) supplemented with 10% fetal calf serum was used. All batches of FCS were tested to give a high plating efficiency and a low spontaneous induced mutation frequency before using a batch for the mutation assay (Source of FCS: Northumbria Biological Ltd)

2.3 X-irradiation

Cell cultures were irradiated as monolayers in flasks (25 and 75 cm^2) at room temperaure. Flasks were placed in a horizontal position during irradiation. An equal dose was confirmed when using two flasks (25 cm^2) in the irradiation jig using a ferrous sulphate dosimeter The source of X-rays was a Siemens Stabilipan set operated at 250 kV and 14 mA with 0.5 mm Cu filtration. The dose rate of 0.75 Gy/min was confirmed using a ferrous sulphate dosimeter (Frankenberg, 1969).

2.4 Mutation assay

2.4.1 *General mutation assay*

As any other mutation assay, this assay can be divided into three separate parts:

(a) The first part involves the exposure of cells to a potential mutagen or well defined mutagen e.g X-rays.

(b) The second part is defined as the expression period. This is the time which is required for the genomic damage to effectively alter the gene product which is then assayed for to determine the induced mutation frequency.

(c) The final part involves plating out of the mutagenised population of cells in a selection medium which normally contains a toxic-drug analogue of the normal gene product. A simultaneous viability assay is also carried out at this point to allow the expression of mutation as the induced mutation frequency per survivor. These steps have been summarised in figure 2.1.

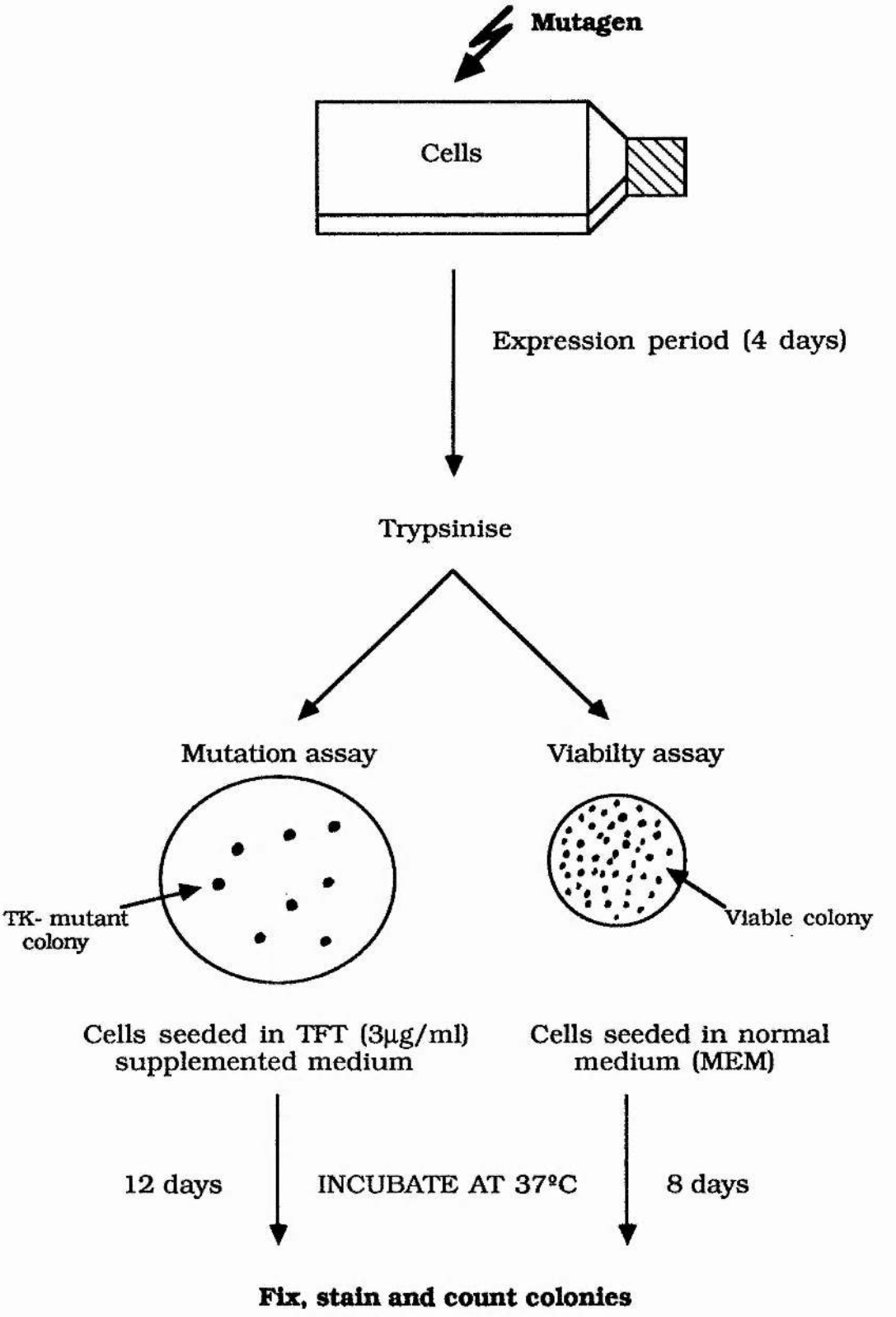


Figure 2.1. Summary of the steps involved in the isolation of *tk*- mutant cells following treatment of hamster cells with a specified mutagen.

During the course of the project, different treatments with the mutagens were carried out hence each treatment is described in the respective chapters unless otherwise mentioned. However, the procedure following the treatment was nearly identical in all the experiments hence is described below.

Following the exposure of the cells to the mutagen, the cells were seeded in 75 cm^2 flasks (Sterlin) and incubated at 37°C for 4 days expression time. This time was found to represent the optimum time for the recovery of *tk*- mutant cells. During the course of the expression period, cells were maintained in the exponential phase of growth by passaging confluent cell cultures. After the expression period, cells were trypsinised and plated out at a concentration of 10^5-10^6 cells per dish (10 cm, Sterlin) with 10 ml of MEM/TFT, the TFT diluted down from the stock to the working concentration of 3 μg/ml. This concentration was found to maximize the recovery of the *tk*- mutant cells. Dishes were incubated for 12 days in an humidified incubator at 37°C with 5% CO_2 in air. After this period, colonies were fixed and stained with Giemsa stain (BDH Ltd) before counting the number of TFT^r colonies.

2.4.2 *Calculation of induced mutation frequencies*

Mutation frequencies in the control cell populations were calculated as follows:

$$M_o = \frac{N_o}{V_o}$$

Where N_O is the number of spontaneously induced mutant colonies in the control cell population, V_O is the number of viable cells in the same unirradiated population at the time of mutant selection, M_O is the mutation frequency per viable cell (or per survivor) in the unirradiated population.

Similarly, the mutation frequency in the treated cell populations were calculated as follows:

$$M_x = \frac{N_x}{V_x}$$

Where, N_X is the number of mutant colonies in the treated cell population, V_X is the number of viable cells in the same treated cell population determined at the time of mutant selection, and M_X is the induced mutation frequency per viable cell (or per survivor) in the treated cell population.
Hence the net induced mutation frequency is given by:

$$M_X - M_O$$

2.4.3 *Viability assay*
After the 4 days expression period, trypisinsed cell suspensions were diluted down in MEM/FCS to give 100-200 cells per 5cm dish supplemented with 5 ml MEM/FCS. Dishes were incubated for 8 days in an humidified incubator at 37°C with 5% CO_2 in air before fixing and counting the number of viable colonies.

2.5 Survival assay

Assay of survival was performed in order to determine the relative X-ray sensitivity alone or in combination with other treatments. For this, sub-cultures of 2 x 10^5 cells in 5 ml MEM/FCS were set up in 25 cm^2 tissue-culture flasks. Flasks were irradiated at a dose rate of 0.75 Gy/min to attain the required dosage. Immediately after irradiation, cells were appropriately diluted and plated out in 5 cm petri dishes (Sterlin) with 5 ml of fresh MEM/FCS. For each dose point, at least three dishes were plated. The dishes were incubated in an humidified incubator at 37°C with 5% CO_2 in air for 8 days. After the removal of medium, colonies were fixed and counted as described in section 2.3.4.

Note: For all experiments, it was important not to move dishes while incubating during colony formation since this would add to the formation of satellite colonies which arise as a result of migrating cells which reattach at a different part of the dish giving rise to a colony.

2.6 Fixing and staining colonies

Medium was poured out of dishes followed by gently rinsing twice with buffer pH 6.4 (Sorenson buffer). After allowing dishes to air dry, colonies were fixed by methanol for 15-30 minutes. Dishes were allowed to dry before addition of a concentrated solution of Giemsa stain which was left for 30 minutes. The stain was washed off by gently dipping dishes in water and allowed to dry before counting the number of colonies. For all clonogenic assay (mutation, viability and survival), colonies with more than approximately 50 cells were scored. Initial observations of colony size were determined under an inverted microscope after which colonies were scored (> 50 cells) based on experience.

2.7 Isolating mutant colonies

Medium from dishes was slowly aspirated and colonies rinsed twice with serum-free medium to remove loose cells which arise as a result of moving the dishes from the incubator. A distinct colony was encircled with a marker pen on the bottom of the dish. Each colony was removed by simply scraping the encircled colony with the tip of a Gilson pipette while simultaneously applying a suction action. This method has been found to effectively isolate cells with little damage. The cell suspension resuspended in 25 cm^2 flasks with 5 ml MEM/FCS. Flasks were incubated at 37°C to allow the formation of a cellular monolayar after which cells were frozen and stored in liquid nitrogen.

Out of the mutant colonies isolated, two *tk*- mutant clones were subsequently used to represent prototype *tk*- mutant cells:

(a) **TK4**: A *tk*- mutant cell line isolated following exposure of CHO KI cells to 4 Gy of X-rays

(b) ***xrs* (*tk*-)**: A *tk*- mutant cell line isolated following exposure of *xrs* 5 cells to 2 Gy of X-rays.

2.8 Freezing cells for storage

Exponentially growing cells were trypsinised and ressupeneded in MEM/FCS to give a high cell concentration (10^6-10^7 cell/ml). Suspensions were pipetted three to four times to give a single cell suspension. Cells were resuspended in Dimethysulfoxide (DMSO) to give a final DMSO concentration of 10%. Samples (1 ml) were transferred into freeze vials, capped and immediately transferred to a -70°C freezer. The vials of the frozen cells were transferred to a liquid storage tank after 24 hr.

CHAPTER 3

Optimum conditions for the mutation assay

3.1 Introduction

This chapter is broadly divided into two parts. The first describes development of conditions necessary for the induction and the subsequent selection of TFT-resistant mutants (*tk*-) while the second part describes experiments and results to confirm the loss of activity of the gene product i.e. thymidine kinase in the isolated *tk*- mutant clones thus confirming a genetic basis for the selected TFT-resistant colonies representing the *tk*- phenotype. For all mammalian mutation assays currently used, it is important to optimize conditions for a particular system due to the variables involved e.g. growth medium, serum batch and differences due to cell type. There is increasing evidence suggesting that inadequate conditions for both cell culture and mutant selection are responsible for the conflicting data produced in different laboratories (DeMars, 1974., Siminovitch, 1976). Hence it was important to establish the optimum conditions for the CHO/*tk* mutation assay since previous studies (Clive et al, 1972., Roufa et al, 1973) have found the isolation of CHO *tk*- mutants difficult in cultured mammalian cells. This is due to the homozygous nature of most autosomally located genes such as the *tk* in the parent wild-type cell lines, thus making the isolation of recessive mutations very difficult in diploid cells. This in turn has limited the number of biochemical studies using *tk*- mutant phenotypes (Clive and Voytek, 1977., Clive et al, 1979). Despite the above drawbacks of using autosomal loci, some studies have successfully isolated recessive mutations using repeated mutagenic and selective steps (Kit et al, 1963., Littlefield and Sarker, 1964., Breslow and Goldsby, 1969., Chu and Ho, 1970). Some of this work is made easier due the high degree of aneuploidy in the hamster cell lines which has resulted in naturally occuring cell lines which are hemizygous or heterozygous for the *tk* locus (Siminovitch, 1976). This hemizygosity could be distributed throughout the genome or restricted to a few chromosomes.

Although wild-type CHO cells are diploid in nature, Deaven and Peterson (1973) suggested that some lines may not be functionally diploid and that some of the genes are present in the hemizygous state. Such a functional hemizygosity of certain genes could have resulted during the evolution of the CHO cell line, hence the karotype

has evolved as a result of chromosomal changes and nucleotide rearrangements. There is increasing evidence that the CHO cells have become functionally hemizygous for certain genes during evolution of this line in cell culture. In contrast to the limited data using the *tk* locus, most of the mutation data involves genes on the X-chromosome which is functionally monosomic in diploid cells irrespective of their sexual origin according to the X-chromosome inactivation theory (Russell, 1961).

For accurate quantitative determination of mutation induction measured by resistance to a drug analogue, in this case TFT resistance, the optimum conditions for the isolation of stable *tk*- mutants had to be established before the induced mutation frequencies could be validly assessed. Three main factors are found to influence the recovery of presumptive mutants in any mutation assay namely: expression time, cell density and concentration of the selection drug (Chu and Malling, 1968).

(a) *Expression time*

This is defined as the time after treatment with a mutagen required for the cytoplasmic dilution of the gene product in the cytoplasm (in the present case, the enzyme thymidine kinase) hence maximizing the recovery of mutant cells. This time delay (number of cell divisions after treatment) is important since the wild type cell population have the enzyme remaining from the premutated condition which must be used up to give rise to an enzymeless cell population. During this period, the damage induced in the mutant cells is fixed in the DNA and the level of the wild-type enzyme (thymidine kinase) and mRNA coding for the enzyme decreases to allow maximum expression of the mutant phenotype. It is important to determine the optimum expression time since some earlier studies on mammalian cell mutagenesis showed that the induced mutation frequency follows a typical pattern i.e. an increase-optimum-decrease (Bridges and Huckle, 1970., Cambray et al, 1987., Chu and Malling, 1968) measured as a function of increasing time. The kinetics of the expression time curve pose a problem to determine the right expression time for a

particular mutation assay. An early expression time would result in an underestimation of the induced mutation frequency while a longer expression time results in the mutation frequency being deduced from the 'decreasing' part of the curve where the viability of the mutant population is decreasing due to the selective disadvantage of the mutant cell population compared to the wild type non-mutated population.

To overcome these problems, it has become important to define the expression time for a specific mutation assay with the use of appropriate experimental schedules (Abbondandolo et al, 1976., Carver et al, 1976., Fox, 1975., Hsie et al, 1975., Myhr and Di Paolo, 1975., Van-zeeland and Simons, 1976). Another problem associated with expression time is its dependence on dose (Arlett and Harcourt, 1972a) which would give rise to different experimental schedules for cells exposed to different doses of radiation.

Currently, the most widely used mutation assay involves resistance of Chinese hamster V79 cells to 6-thioguanine measuring induced mutation frequencies at the *hprt* locus. Despite the extensive mutation data involving this locus, the different optimum expression times used (table 3.1), highlight both the problems and importance to determine the optimum expression time for a specific mutation assay in a particular laboratory.

Cell line	Target locus	Expression time (days)	Reference
V79	*hprt*	2	Thacker et al, 1977
V79	*hprt*	7	Nikado and Fox, 1976
V79	*hprt*	8	Vrieling et al, 1985
V79	*hprt*	9	Fuscoe et al, 1986

Table 3.1. The expression time used in the Chinese hamster V79 cells to isolate mutants at the *hprt* locus in different laboratories.

(b) *Cell density*

The number of cells plated out in the selection medium have been known to influence the recovery of presumptive mutant cells (Chu and Malling, 1968). In an early investigation, Harris (1968) observed an increase in the frequency of spontaneously induced mutations in pig kidney cells (measured by resistance to puromycin) with a corresponding increase in number of cells seeded in the selection medium. He attributed this to a 'feeder-layer effect' in which the high cell density was thought to provide conditions which enhanced both the growth and recovery of mutant cells. However, evidence against this hypothesis was provided by Chu and Malling (1968) who showed that the number of mutants detected per dish decreased beyond a certain cell concentration. This suggested that the crowded conditions in the selection medium rather provide a 'feeder-layer effect' impaired the recovery of mutant cells.

This cell-concentration dependent phenomenon has been suggested to be as a result of metabolic co-operation, an energy independent process (Cox et al, 1972) between the mutant and wild type cells which reduces the recovery of the potential mutants (Subak-Sharpe et al, 1969). This phenomenon is thought to arise as a result of cell-cell contact observed at high cell densities (Cox et al, 1970) which has been shown to facilitate the exchange of nucleotides, mRNA

or enzymes across the cell membrane (Ashkenazi and Gartler, 1971., Cox et al, 1970, 1972., Fujimoto and Seegmiller, 1970). During the cell contact, evident at high cell densities, the mutant cell would regain some of the gene product from the wild type cell population. This would allow the mutant cell to incorporate the toxic selection drug as a result of the transferred enzyme which would lead to its cell death, thus giving rise errors in the estimation of the induced mutation frequency.

(c) *Selection drug concentration*

The optimum drug concentration is one which selectively kills wild-type cells while allowing mutant cells to survive with a high efficiency. The use of too high a concentration would result in death of mutant cells as a result of drug toxicity, while a low concentration would allow the growth of wild-type cells. This would result in a decreased viability of the mutant cell population due to their selective disadvantage. Both the type and concentration of the selection drug should rapidly inhibit cell division in the non-mutagenised cell population to eliminate the process of metabolic co-operation.

3.2 Materials and methods

3.2 (a) Optmum conditions for the CHO/*tk* mutation assay

This section describes experiments performed to determine the three main parameters described above which influence the recovery of mutant cells: expression time, TFT concentration and cell density in selection medium.

3.2.1 *Optimum expression time*

To determine the optimum expression time, four independent samples were set up which included a control (unirradiated) and three samples exposed to different X-ray doses (2,4 and 6 Gy). For each sample, six 75 cm^2 (Sterlin) flasks were set up with densities of CHO KI cells ranging from 2 $x10^5$-2 x 10^6 cells per flask, the number being

dependent on both the dose and expression time after which cells are plated in the selection drug. Cells were exposed as monolayers to varying X-ray doses as already described in section 2.3. One flask from each sample was plated out immediately after irradiation for the mutation assay (section 2.4) while others were incubated at 37°C for the different expression times (1-5 days) before trypsinizing and plating out in TFT/MEM and MEM/FCS (for the viability assay) to express the induced mutation frequencies as a function of expression time.

3.2.2 *Determination of optimum TFT concentration for selection of tk-mutant cells:*

The optimum TFT concentration is represented by a minimum concentration which effectively eliminated the growth of the parent CHO KI cells. Hence, an experiment was performed to determine the relative cloning efficiency of CHO KI cells as a function of TFT concentration. For all the experiments, TFT was freshly prepared and protected from fluorescent light using aluminium foil. The stock solution of TFT (1 mg/ml) was diluted down to give the various final concentrations ranging from between 0.01-30 μg/ml in normal MEM/FCS medium. Exponentially growing CHO KI cells were trypsinised and diluted down to give approximately 200-6000 cells per 10 cm dish (Sterlin). The number of cells plated per dish was dependent on the concentration of TFT e.g 200 cells/plate with 0.01 μg/ml and 6000 cell/plate with 30 μg/ml TFT/MEM. Triplicate plates were seeded for each dose point which were incubated for 10 days at 37°C in a humidified incubator with 5% CO_2 in air. Dishes were fixed and stained (section 2.7) before evaluating the relative cloning efficiency of CHO KI cells as a function of the TFT concentration.

3.2.3 *Optimum cell plating density*

To determine the optimum cell plating density in the selection medium (TFT/MEM), a *tk-* mutant cell line isolated in a preliminary experiment designated as TK4 (described in section 2.7) was used as a prototype *tk-* mutant cell for a reconstruction experiment.

Exponentially growing TK4 cells (maintained and passaged in MEM/FCS) were seeded at 150 cells per 10 cm petri dish (Sterlin) in the presence of increasing concentration of CHO KI wild type cells (10^3-10^7). The dishes were supplemented with 10 ml TFT/MEM medium (TFT: 3µg/ml) and incubated in an humidified incubator at 37°C for 10 days before fixing and counting the number of mutant colonies. As a control, 150 TK4 cells alone were seeded in MEM/FCS and TFT/MEM medium.

3.2 (b) Analysis of *tk*- mutants

This section describes experiments performed to confirm the loss of thymidine kinase activity in the *tk*- mutant cells. In all the experiments described below, the mutant line TK4 was used to represent a 'true' *tk*- mutant phenotype unless otherwise indicated.

3.2.4 *DNA synthesis assay*

Unlabelled exponentially growing CHO KI and TK4 cells were diluted down to a concentration of 5 x10^5 cells/ml. From this dilution, 1 ml of cell suspension was placed in V-tubes (Sterlin) and incubated in waterbath set at 37°C and allowed to equilibrate for 30 minutes (5 tubes set up for both cell lines). In all except the control tubes, 100 µl (1µCi) of ^{3}HTdR was squirted into cell suspension and incubated for various times (10-40 minutes) to allow uptake of radioactivity. After the required incubation time, the reaction was terminated by the addition of 5 ml ice cold saline forcefully to each sample placed on ice. When all the samples had accumulated on ice, the tubes were centrifuged, the supernatent aspirated and pellet vortexed. This was followed by addition of 1 ml of 0.03M NaOH to lyse the cells followed by 1.5 ml of 0.62M Trichloroacetic acid (TCA) 10 minutes later. Samples were stored overnight at 4°C to allow precipitation of DNA. The samples were vortexed before collecting DNA onto a 25 mm glass fibre-filter (Whatmann) soaked in 0.31M TCA solution using a suction unit. Filters were rinsed twice with ice-cold 0.31 M TCA and once with ice-cold ethanol before transferring into to scintillation vials. To each vial, 4 ml of scintillation cocktail (Optiphase MP, LKB) was

added, samples mixed and the radioactivity per filter determined using a LKB 1214 Rack beta liquid scintillation counter.

3.2.5 *Spontaneous reversion of tk- mutants*

Exponentially growing TK4 mutant cells were trypsinsed and diluted down in MEM/FCS to give approximately 10^2-10^6 cells per 10 cm perti dish supplemented with 10 ml HAT/MEM medium for reversion analysis. As a control, 200 TK4 cells were plated in MEM/FCS to determine the number of survivors. As a second control to check for the selection conditions, 200 CHO KI cells were plated in HAT/MEM medium in 5 cm dishes. All dishes were incubated for 8 days before fixing and counting the number of tk^+ revertant colonies.

3.2.6 *Growth characteristics of tk- mutant cells in comparison to CHO KI cells*

Exponentially growing TK4 and CHO KI cells were trypsinised and cell concentration determined. Cells were seeded at approximately 10^4 cells per 5 cm petri dish supplemented with 5 ml of either normal medium (MEM/FCS), TFT/MEM or HAT/MEM and incubated at 37°C. After every 24 hr, cells from one dish were trypsinised and the cell concentration determined.

3.2.7 *Stability of mutant cells (colony assay)*

Both TK4 and CHO KI cells were seeded at 200 cells per 5 cm petri dish in the presence of either normal or TFT/MEM medium. Dishes were incubated for 8 days at 37°C before fixing, staining and counting the number of viable colonies.

3.2.8 *Autoradiography*

The role of the thymidine kinase in the uptake of extracellular thymidine during DNA synthesis formed the basis of a sensitive test to determine the loss of enzyme activity. Uptake of a radioactively labelled nucleotide analogue (^{3}HTdR) during DNA synthesis visualised

by the formation of silver grains in a photographic emulsion was used to verify the presence/absence of thymidine kinase activity in the *tk-* cells.

3.2.8 a *Labelling cells with radioactivity*

For each cell line (TK4 and CHO KI), three 25 cm^2 flasks were seeded at a density of approximately 2 $x10^5$ cells. Flask were labelled as control, TFT + ^{3}HTdR and MEM + ^{3}HTdR samples and incubated overnight at 37°C in 5 ml medium. A stock solution of ^{3}HTdR was diluted down in distilled water to give a concentration of 0.1 µCi/µl. To each flask (except control) 10 µl of the diluted radioactivity was added to give the final working activity of 1µCi. The flasks were gently shaken to allow the radioactivity to mix before further incubating for 24 hr at 37°C to allow uptake of the label.

3.2.8 b *Preparing slides for autoradiography using the cytospin method*

Cells were trypsinised and resuspended in fresh MEM (without serum) and centrifuged for 5 minutes at 1000 rpm. Supernatent was aspirated and pellet resuspended by tapping V-tubes (repeat twice). Cells were finally resuspended in MEM (without serum) to give a final concentration of 10^6 cells/ml. Samples were loaded into cytospin buckets and spun in cytospun for 10 minutes at 800 rpm (Shandon, Cytospin Model II). Slides were allowed to dry in air before fixing for 10 minutes in methanol.

3.2.8 c *Developing and fixing*

This procedure was carried out in a dark room with a 15 W lamp covered with a light brown safelight filter (Ilford 902). K2 Emulsion in gel form (Ilford Nuclear Research) was placed in a 50 ml measuring cylinder and melted by incubating in a waterbath set at 45°C. The amount of emulsion dissolved depended on the number of slides to be dipped (in most case, a final diluted volume of 40-50 ml was found to be sufficient). After a period of 20 minutes (time required for the emulsion to melt), an equal volume of 1% glycerol was added and

mixed with the emulsion by pouring it back and forth into a clean staining jar. This solution was further incubated in the waterbath for an additional 15 minutes to remove any air bubbles present in the emulsion solution before pouring into a clean staining vessel.

Each slide was dipped once for about 1 second in the emulsion, the reverse side wiped with a clean tissue and stood upright against the wall to allow the slide to dry. After about 4 hr (time for emulsion to dry), the slides were placed in a light proof box which contained silica gel to prevent dampness from damaging the emulsion. The box once sealed was further covered with a polyethylene black bag to prevent exposure to light. The box was refrigerated at 4°C for a 4 day exposure period.

After the exposure time, the slides were brought back to the dark room and allowed to come back to room temperature. This was important since the optimal temperature of the Kodak D19 developer is about 20°C. The fixative used was Kodak Acid Fix (Hypo) which was diluted 3 times in distilled water. The slides were developed for 3.5 minutes, rinsed for 30 seconds in distilled water and fixed for 5 minutes. This was followed by a gentle rinse for 20 minutes in running tap water and stained according to the Jenner/Giemsa technique described below.

3.2.8 d *Jenner/Giemsa staining*

Slides were transferred to a staining jar containing Jenner stain freshly diluted with an equal volume of buffer solution (pH 6.8). Slides were allowed to stain for 5 minutes after which they were transferred without washing to a jar containing Giemsa stain freshly diluted with nine volumes of buffer solution. After being stained for 10-15 min, the slides were transferred to a jar containing buffer solution and rapidly washed in three or four changes of water and allowed to dry. Once totally dry, the slides were covered with coverslips mounted with Euparal.

3.2.9 *5-azacytidine (AC) reversion experiments*

This assay was based on protocol described by Jeggo and Holliday (1986) to test for any azacytidine-induced reactivation of a possible

methylated copy of the *tk* gene. To induce reversion by 5-azacytadine (Sigma), both CHO KI and a *tk-* mutant cell line (TK4) were seeded at 1×10^5 cells per 25 cm^2 flasks (Sterlin) and incubated overnight. AZ is unstable in aqueous solution; therefore it was prepared immediately before use at a concentration of 1 mg/ml. This was added to the flasks to give a final concentration of 1μg/ml and incubated at 37°C for 15 hrs. The medium was changed after this period and replaced with fresh MEM/FCS. Cultures were maintained in exponential growth for an additional 5 days (passaging during this time if the cultures became confluent) before plating out 150-200 cells per 5 cm dishes in the presence of either normal or HAT/MEM. Dishes were incubated at 37°C for 10 days.

3.3 Results

3.3.1 *Expression time*

The results of the induced mutation frequency expressed as a function of expression time (days) are presented in figure 3.1. The three curves represent the induced mutation frequencies in the parent CHO KI cell line following an exposure to 2,4 and 6 Gy X-rays. All the data points represent the induced mutation frequency after subtraction of the spontaneously induced mutation frequency.

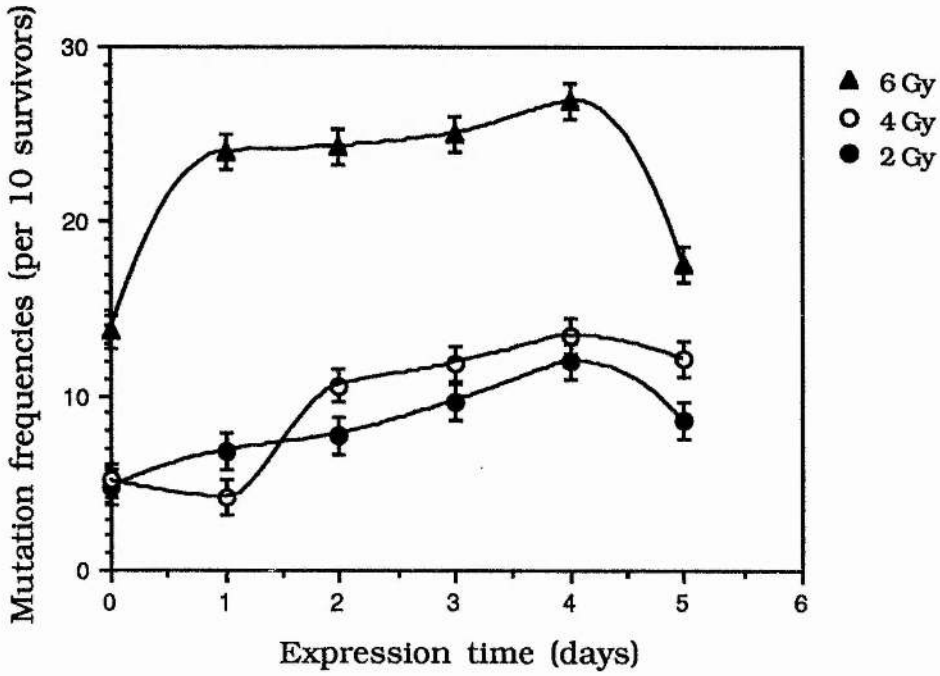


Figure 3.1. Mutation frequency as a function of expression time in CHO KI cells exposed to 2,4 and 6 Gy of X-rays. Vertical bars represent the standard errors of the mean values from three independent experiments.

The curves obtained follow a typical pattern as observed by previous workers (Chu and Malling, 1968). The expression-mutation relationship curves can be subdivided into three parts: an initial increase, a peak followed by a reduction in mutation frequency. Based

on these experiments, 4 days was used to represent the optimum expression time required for the complete 'dilution' of any cytoplasmic thymidine kinase hence maximize the recovery of persumptive *tk*- mutant cells.

3.3.2 *TFT concentration*

The relative cloning efficiency of the CHO KI cell line in the presence of increasing TFT concentration in the selection medium (TFT/MEM) is presented in figure 3.2.

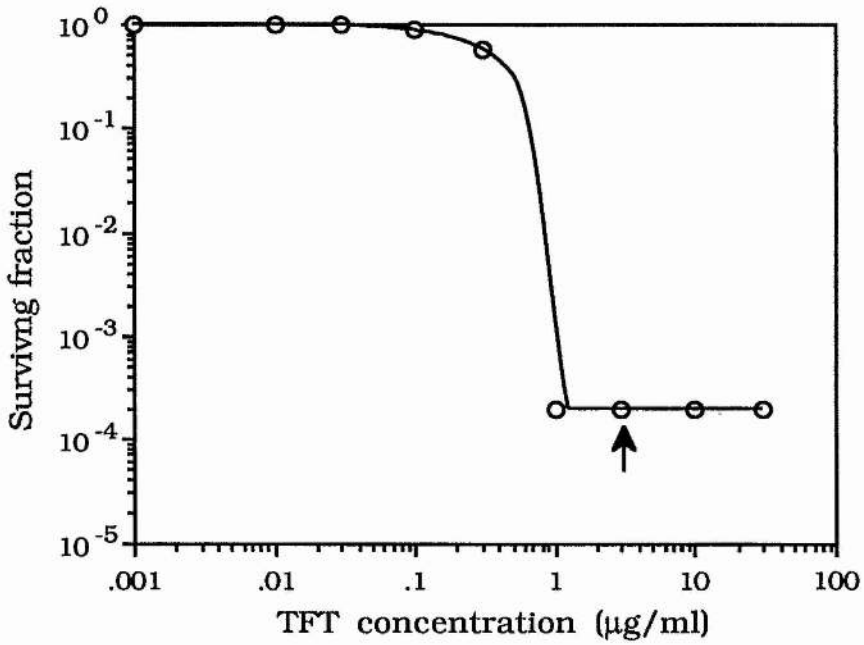


Figure 3.2. Relative cell survival of CHO KI cells as a function of TFT drug concentration. Each point represents a survival determination from the mean three independent experiments.

A concentration of TFT in the range of 0.001-0.2 μg/ml permits growth of CHO KI cells. However, increasing the concentration to the range of 1μg/ml results in the complete loss of cell growth. This complete loss of survival is also observed at the higher concentrations (1-30μg/ml). Hence for all the mutation experiments, an intermediate

concentration of 3μg/ml was used. The use of a too low concentration of TFT (below 1μg/ml) would result in unacceptably high levels of false positive results i.e. non-mutant cells forming colonies while a high concentration would result in the assay becoming less sensitive due to the the toxic effects of the drug.

3.3.3 *Cell density*

The relative cloning efficiency of the *tk*- mutant cell population (TK4) in the presence of increasing concentrations of the CHO KI cells (wild-type cell population) is presented in figure 3.3.

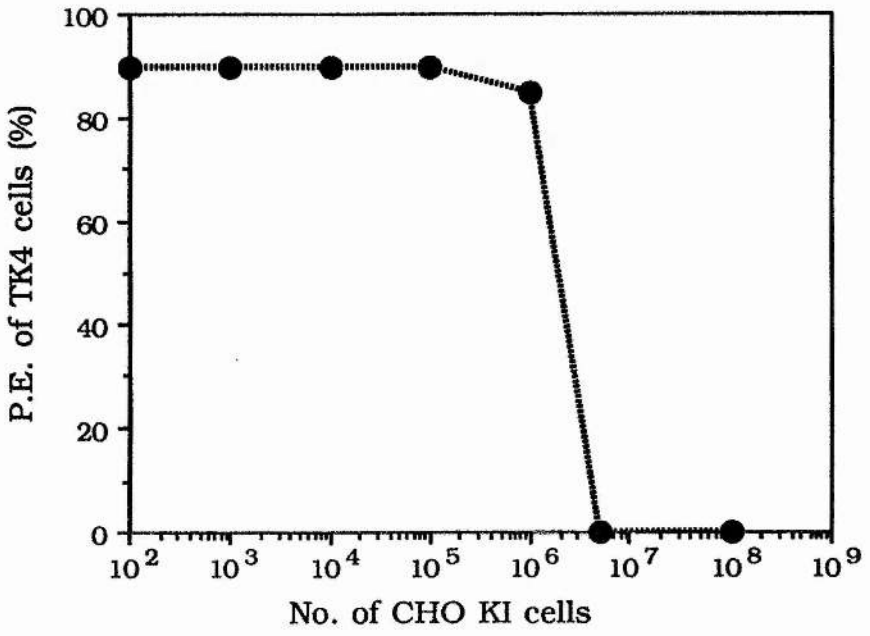


Figure 3.3. Effect of CHO KI cell concentration on the recovery of TK4 mutant cell line.

A near constant P.E of TK4 cells is observed (about 90%) in the presence of CHO KI cell concentration ranging from between 10^3-10^6. However, a sharp decline is observed once the cell concentration exceeds 10^6 cells per dish. For all the experiments described in this thesis, a standard cell density range of between 10^5-10^6 cells per dish

(1300-13000 cells/cm^2) was maintained. The cell concentration plated was dependent on the type of cell line used, its radiosensitivity and the type of mutagenic treatment. This concentration range is consistent with some of the earlier reports which have found this to represent the optimum cell density (Adair and Carver, 1979).

3.3.4 *Uptake of ^{3}HTdR in tk- mutant cells*

Both the normal (CHO KI) and mutant (TK4) cells were incubated in the presence of ^{3}HTdR and its uptake via the salvage pathway measured. This is a simple and quick assay to establish the presence/absence of the enzyme thymidine kinase. The amount of ^{3}HTdR activity incorporated into the DNA (in terms of disintergrations per minute measured in the liquid scintillation counter) at various sampling times is shown in figure 3.4.

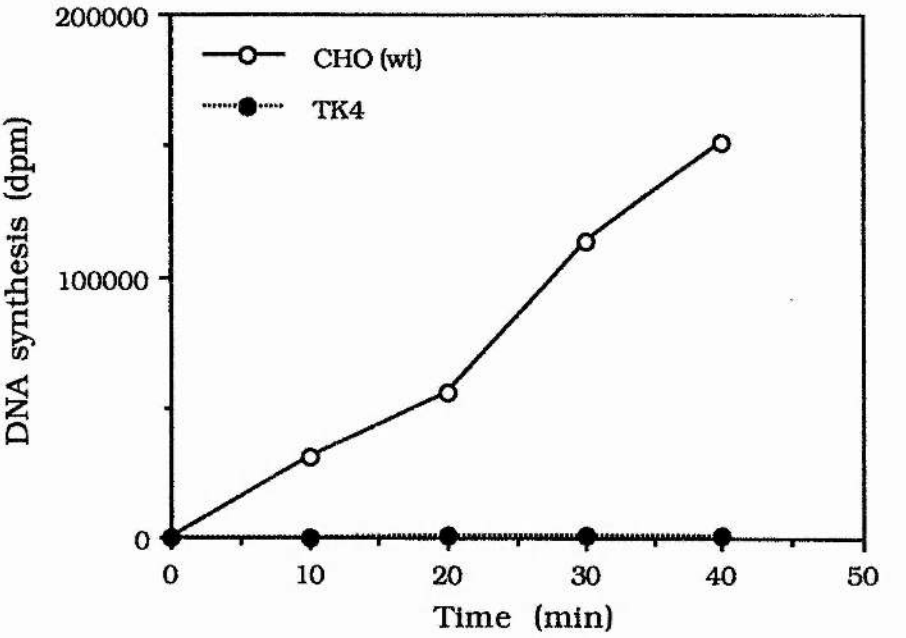


Figure 3.4 DNA synthesis measured as a function of incubation time in the presence of ^{3}HTdR in both the normal CHO KI and TK4 mutant cell lines.

The normal CHO KI cell line shows a linear increase in the uptake of the ^{3}HTdR in comparison to the TK4 cell line which shows a complete lack of incorporated radioactivity. This observation provides evidence for the inability of the mutant cells to incorporate the extracellular source of thymidine principally via the salvage pathway. This suggests that the *tk*- mutants obtained in the CHO/*tk* mutation assay represents mutants with a genetic change at the *tk* locus resulting in the inability of the cells to synthesize the active form of the enzyme thymidine kinase.

3.3.5 *Spontaneous reversion frequencies*

In order to confirm the stability of the mutant cells, a colony assay measuring the spontaneous reversion of mutants was performed. The results obtained are presented in table 3.2.

No. of mutant cells seeded/dish	Plating efficiency (%) in normal medium (MEM/FCS)
200	50
No. of mutant cells seeded/dish	Reversion frequency/survivor in HAT/MEM
10^{3}-10^{6}	$< 10^{-6}$

Table 3.2. The plating efficiency of mutant cell line in normal medium used to express the spontaneous reversion frequencies per survivor measured by plating various mutant cell concentrations in the presence of HAT/MEM selection medium.

For the reversion analysis, the mutant line TK4 was grown in the presence of normal medium for more than 25 passages to allow any reversion to a *tk*+ phenotype before plating out in HAT/MEM to

measure the spontaneous reversion frequency. The apparent absence of any reversion (< 10^{-6}) suggests that the *tk*- mutants have undergone a stable genetic change which further supports the use of the CHO/*tk* mutation assay.

3.3.6 *Autoradiography*

Autoradiography was one of techniques used to verify the loss of thymidine kinase activity in the mutant cells (TK4) based on the cellular incorporation of ^{3}HTdR via the 'salvage' pathway which would require the presence of an active form of thymidine kinase. This technique was specifically used to identify a possible sub-class of *tk*- mutants which might have an altered form of thymidine kinase which would be visualised with percentage of cells showing an uptake of the label in form of the silver grain formation. The results obtained are represented in table 3.3, plate 3.1 and plate 3.2.

Treatment	Labelled cells (%)
CHO KI + MEM (control)	No label
CHO KI + MEM + ^{3}HTdR	100
CHO KI + TFT + ^{3}HTdR	97
TK4 + MEM (control)	No label
TK4 + MEM + ^{3}HTdR	No label
TK4 + TFT + ^{3}HTdR	No label

Table 3.3. The uptake of ^{3}HTdr measured using autoradiography in both the CHO KI and TK4 mutant cell line

The CHO KI cells show a completely labelled cell population (100%) in comparison to the TK4 mutant line in which no incorporation of the radioactive label is evident. For the autoradiographic determinations, the mutant line TK4 and the CHO KI

cell lines were labelled in the presence of TFT/MEM. Plate 3.1 shows the results of CHO KI cells incubated with the radioactivity while plate 3.2 shows the uptake no uptake in the mutant TK4 cells. The CHO KI cell population grown in TFT/MEM show heavily labelled cells despite some cells showing the toxic effects of the drug (distorted cells). Cells with less than 50 grains was the criterea used to represent a cell population as 'no label' (Table 3.3).

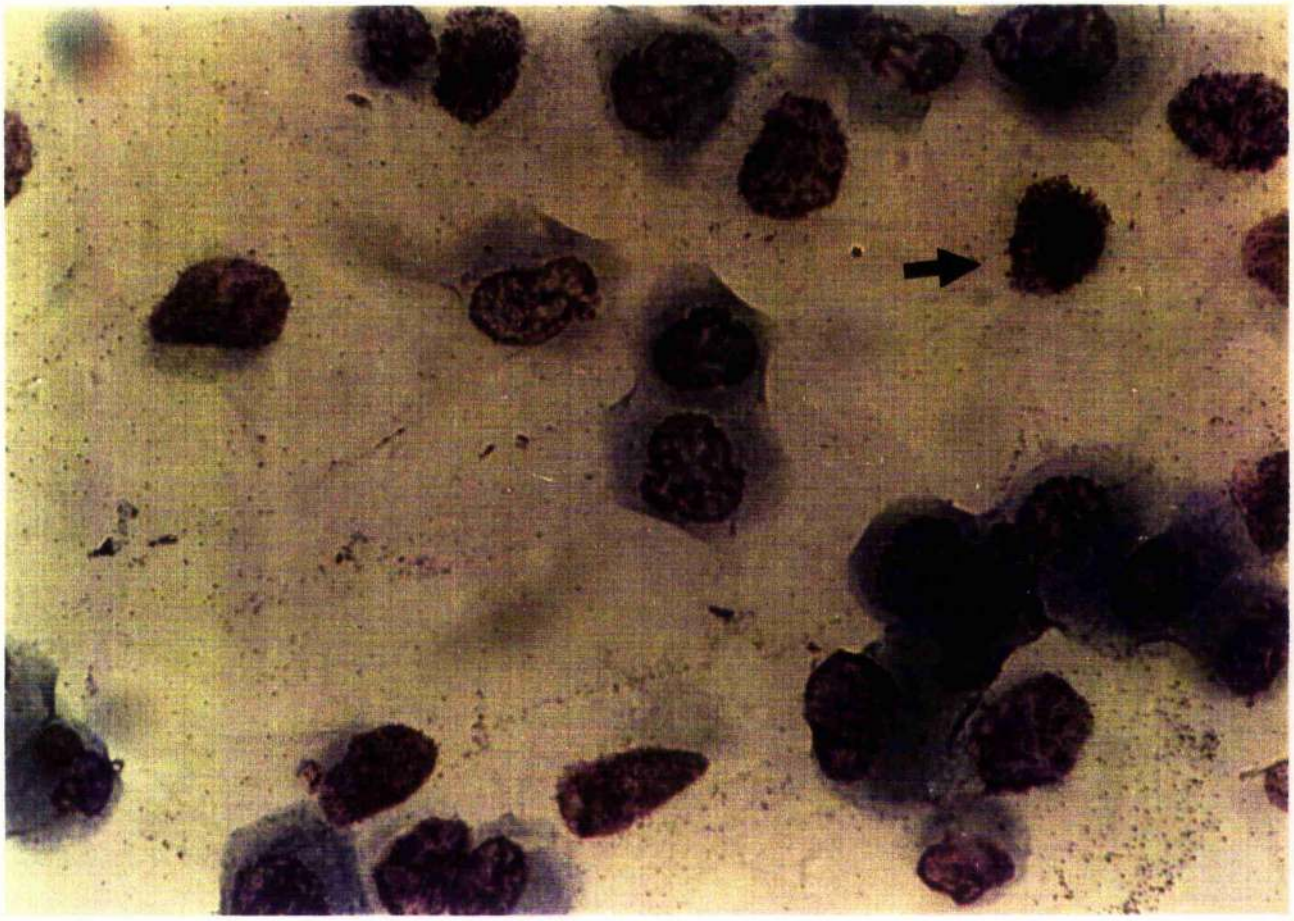


Plate 3.1. Autoradiograph of slide showing CHO KI cells incubated in TFT/MEM medium in the presence of ^{3}HTdR.

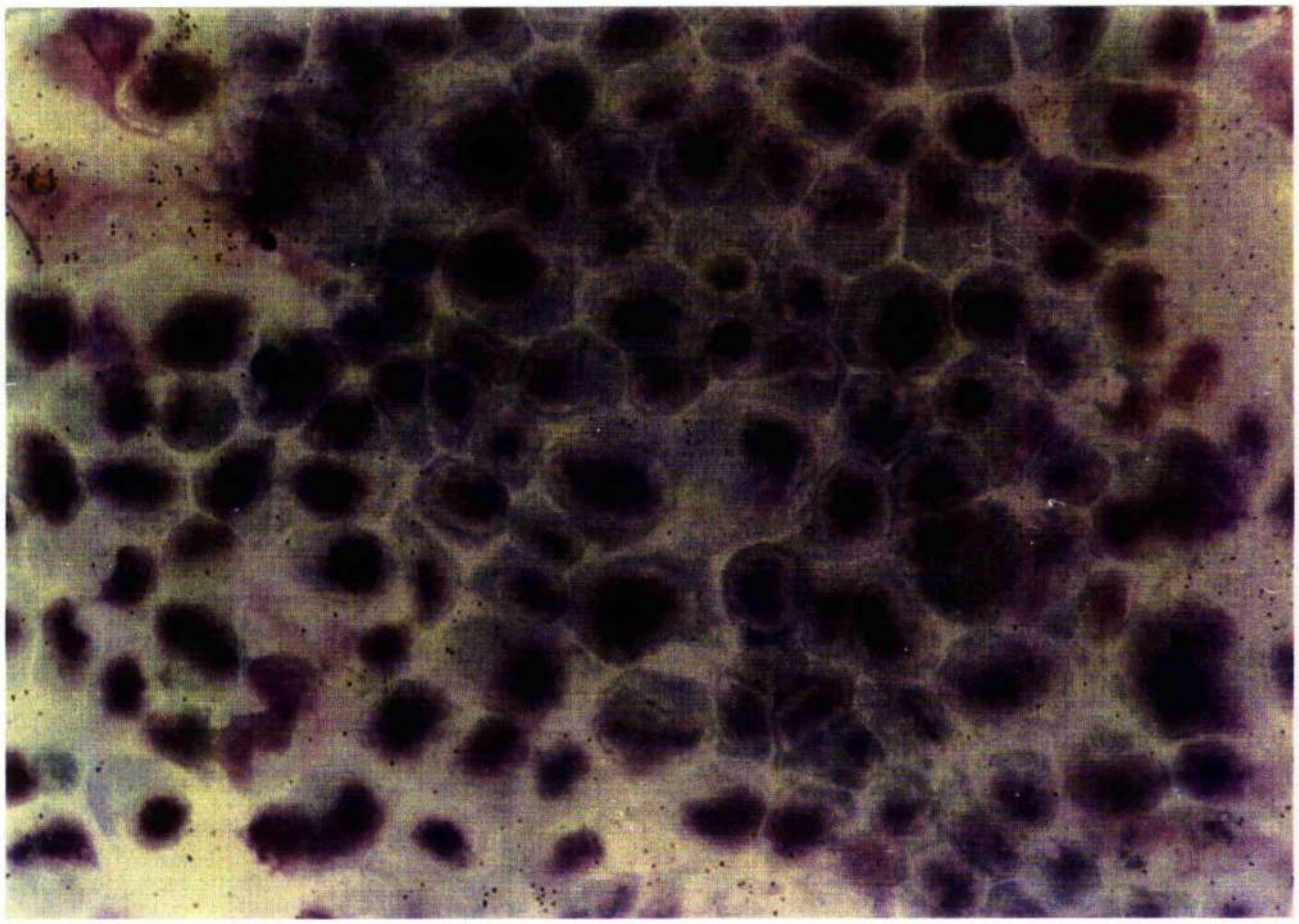


Plate 3.2. Autoradiograph of slide showing TK4 mutant cells (tk-) incubated in TFT/MEM in the presence of ^{3}HTdR

3.3.7 *Growth characteristics*

The stability of a *tk*⁻ mutant was tested by plating both the mutant TK4 and the normal CHO KI cell line in the presence of the three main medium compositions used throughout the course of this project. These are *tk*⁻ selection medium (TFT/MEM), *tk*⁺ selection medium (HAT/MEM) and normal medium (MEM/FCS). The growth curves presented in figures 3.5-3.7 were obtained by counting the total number of cells per dish sampled at various time intervals. Figure 3.5 shows the growth curves of both CHO KI and TK4 cell lines incubated in the presence of MEM/FCS.

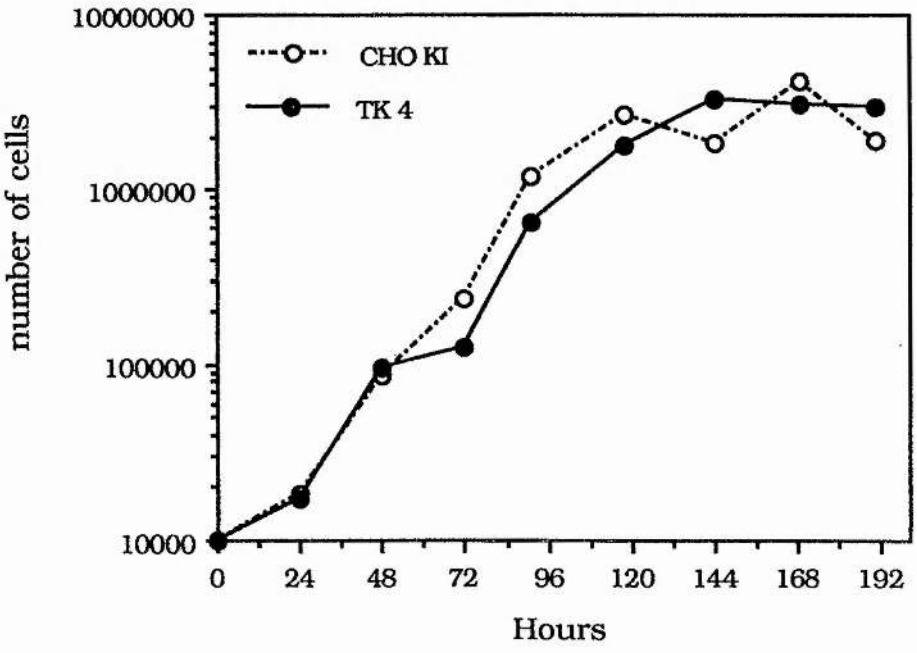


Figure 3.5. Growth curves of CHO KI and TK4 mutant cell line in MEM/FCS medium. Each point represents the average cell count from three separate dishes.

The mutant line TK4 shows a comparable growth curve to that observed in the parent CHO KI cell line and can be maintained in continous culture in normal medium. In contrast to the above results, a different growth pattern is observed when both the normal and mutant cell line are plated in the presence of TFT/MEM shown in figure 3.6.

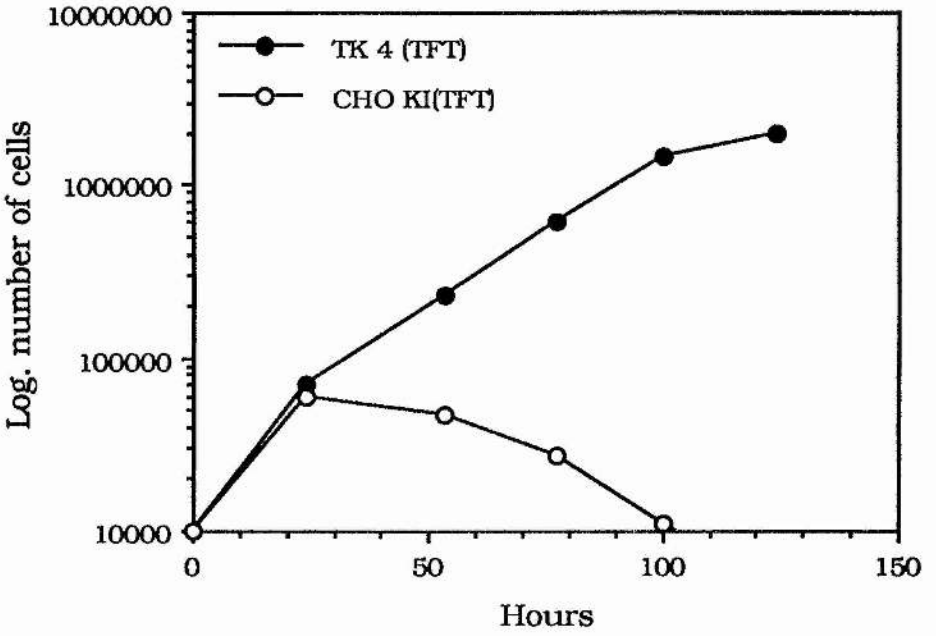


Figure 3.6. Growth curves of CHO KI and TK4 mutant cell line measured in the presence of TFT/MEM.

The mutant line exhibits a normal exponential increase in cell number compared to the complete loss of cell growth observed in CHO KI cells when plated out in the TFT/MEM medium. The growth of the mutant line in the continous selection pressure of TFT/MEM provide evidence for stability of the genetic change in the *tk* locus. To further confirm the stability of the *tk*- mutants in terms of its reversion to the tk^+ phenotype, the growth charactersistics of both the TK4 and CHO KI cell line was followed by plating in HAT/MEM medium. The results obtained are presented in figure 3.7.

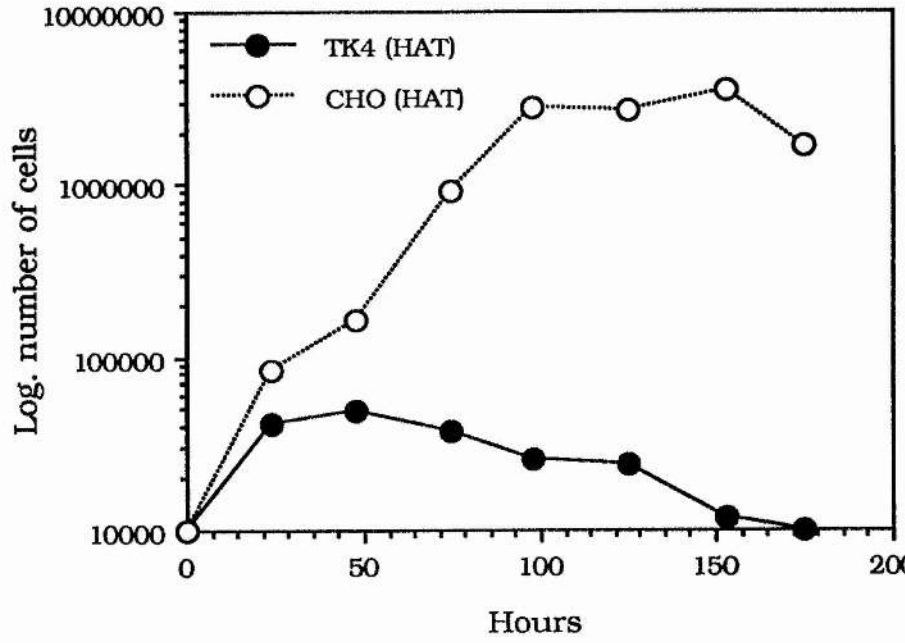


Figure 3.7. Growth curves of both the CHO KI and TK4 mutant cell line in the presence of HAT/MEM.

The CHO KI cell line shows normal growth characteristics in the presence of HAT supplemented medium in comparison to the TK4 cells in which there is a complete loss of cell survival immediately after plating the cells in the presence of HAT/MEM. This is evidence for a genetic change at the *tk* locus as well as a low reversion frequency which is further supported by the results of the colony assay (see section 3.3.5). The growth of the CHO KI cells in HAT/MEM medium validates its use as a counterselective medium to recover tk^+ cells e.g. during the spontaneous reversion experiments (section 3.3.5).

3.3.8 *Plating efficiencies of mutant cell line*

The colony assay results of both the CHO KI and TK4 mutant cell line plated in the presence of MEM/FCS and TFT/MEM medium are presented in figure 3.8

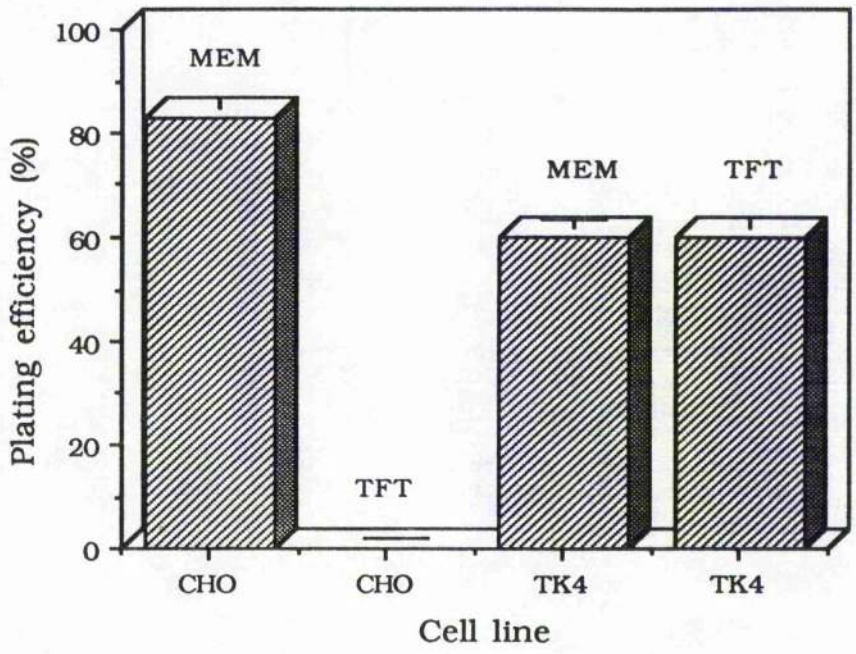


Figure 3.8. The plating efficiencies (P.E) measured in CHO KI and TK4 mutant cell line using a colony assay in the presence of both MEM/FCS and TFT/FCS

The mutant line TK4 shows a reduced P.E (60%) compared to that observed in the CHO KI cell line (80%) when plated in the presence of normal medium. However, in the presence of the selection medium (TFT/MEM), the CHO KI cell line shows complete loss of cell survival while the TK4 cells have a P.E comparable to that when plated in normal medium. This strong inhibition of growth by TFT on the wild type cell population supports the use of TFT as a effective selection drug (Adair and Carver, 1979). The inhibition of cell growth of CHO KI cells in the presence of TFT/MEM is shown in the results from a typical colony assay (plate 3.3 and 3.4). Plate 3.3 shows the surviving colonies of TK4 cells plated in the presence of (a) normal (b) TFT/MEM medium. Plate 3.4 shows a clear plate in which CHO KI cells were plated in the presence of (a) TFT/MEM in comparison to the 80% P.E observed in (b) MEM/FCS.

Plate 3.3. Results from the colony assay in which mutant cells (TK4) were plated in the presence of MEM/FCS (left) and TFT/MEM (right).

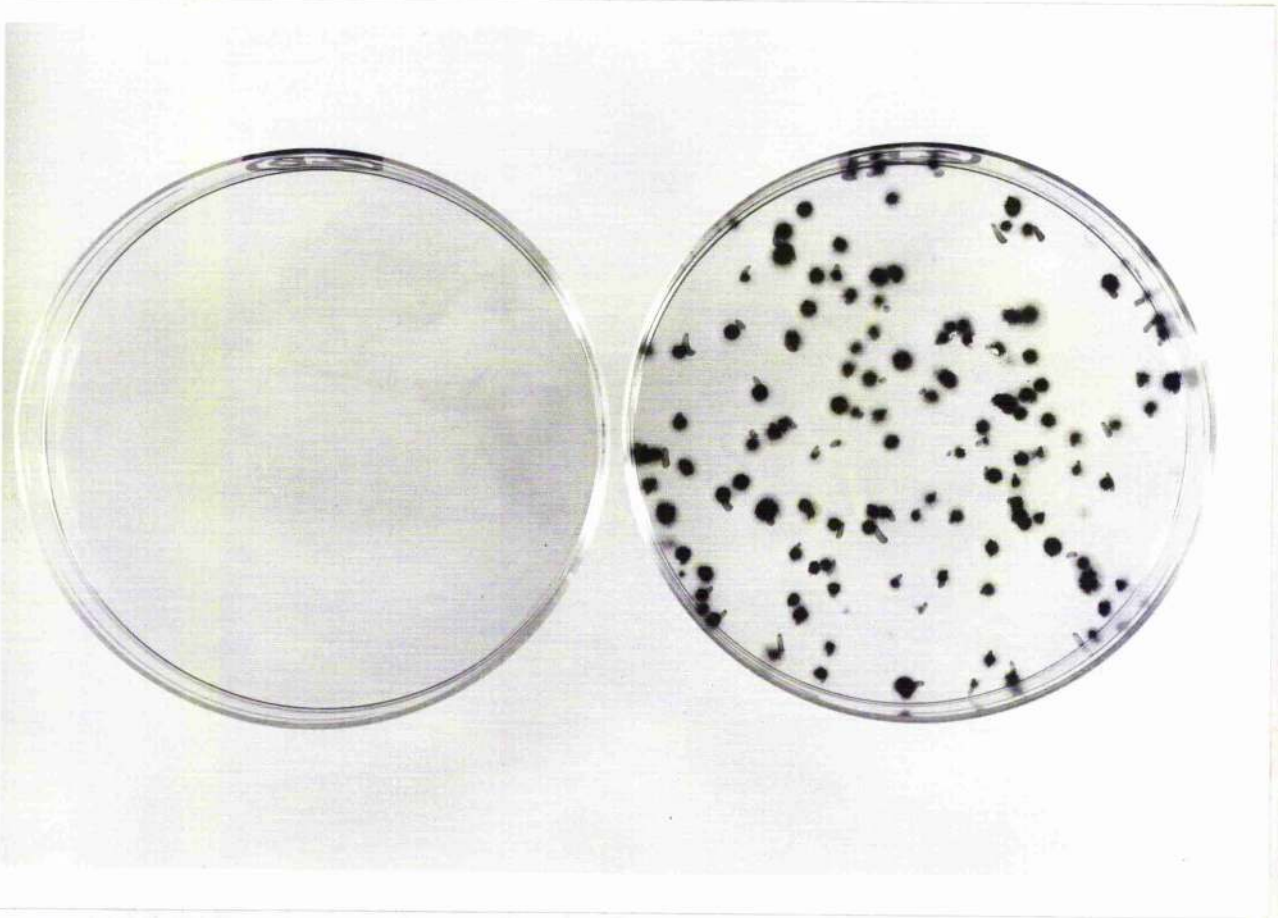


Plate 3.4. Results from the colony assay in which CHO KI cells were plated in TFT/MEM (left) with the complete loss of cell survival compared to cells seeded in MEM/FCS (right) which show the normal P.E.

3.3.9 *Survival assay*

To check for any differences in the radiosensitivity of the mutant line (TK4), a survival assay was performed following the exposure of cells to a graded dose of X-rays (see section 2.4). The survival curves obtained after exposure of both the TK4 and CHO KI cell line to X-rays are presented in figure 3.9.

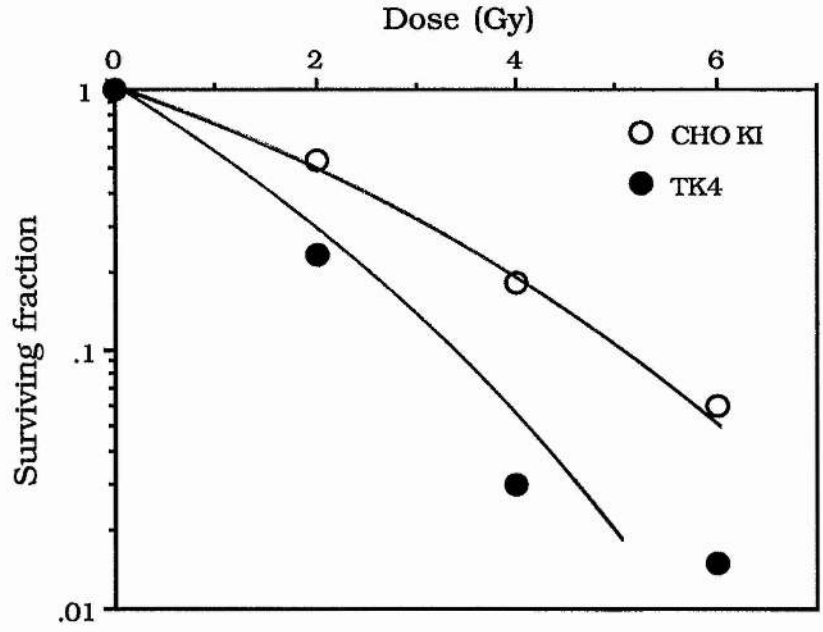


Figure 3.9. Survival curves of TK4 and CHO KI cells exposed to X-rays.

The mutant line shows an increased radiation sensitivity in comparison to the CHO KI cell line. This would suggest that the *tk*- mutants cells may have undergone a mutation which extends beyond the *tk* locus hence affecting essential genes required for cell survival. No conclusions could be made from this result as this could not be repeated (due to the breakdown of the X-ray set in the department).

3.3.10 *5-azacytadine treatment*

The *tk*- mutant cells were treated with the methylating inhibitor agent 5-azacytadine to detect the presence of a possible methylated copy of the *tk* gene which would give rise to epigenetic change at the *tk* locus. Cells treated with AZ were plated in the presence of HAT/MEM. The reversion analysis results carried out with the TK4 mutant cell line are presented in figure 3.10.

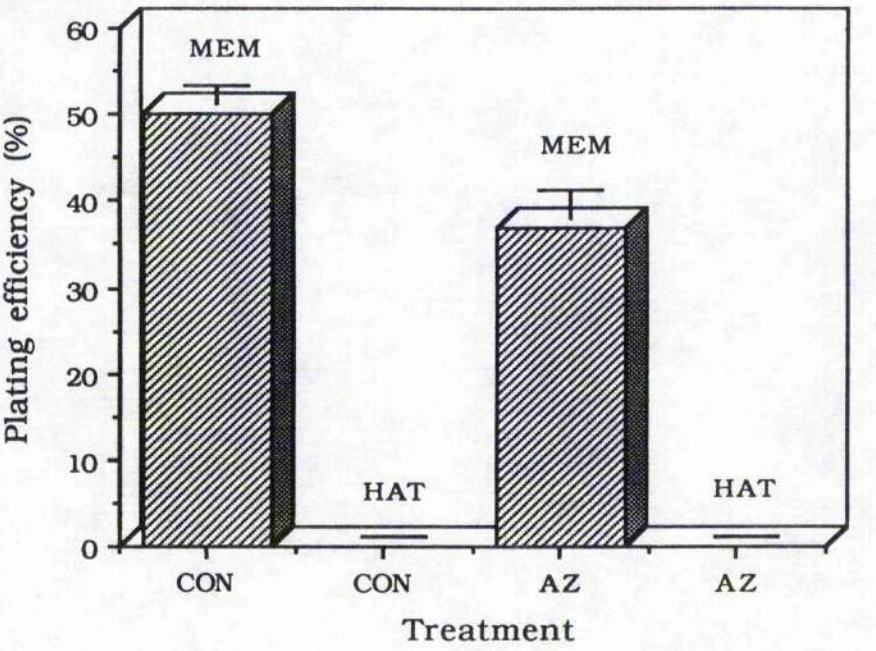


Figure 3.10. The clonal assay following the treatment of the mutant cell line TK4 without (control) or with 5-azacytadine. Cells are plated in both normal medium (MEM/FCS) and HAT-supplemented medium.

The TK4 cells show no cell survival in the presence of HAT/MEM following treatment with AZ in comparison to the average plating efficiency observed in the presence of normal medium in both control and AZ treated cells. The reduced plating efficiency observed in cells treated with AZ is due to the toxic effects of the drug. As a control to check the selection conditions as well to check for any

toxic effects of AZ, the parent CHO KI cells were treated with AZ and plated out in both normal and HAT/MEM supplemented medium (figure 3.11).

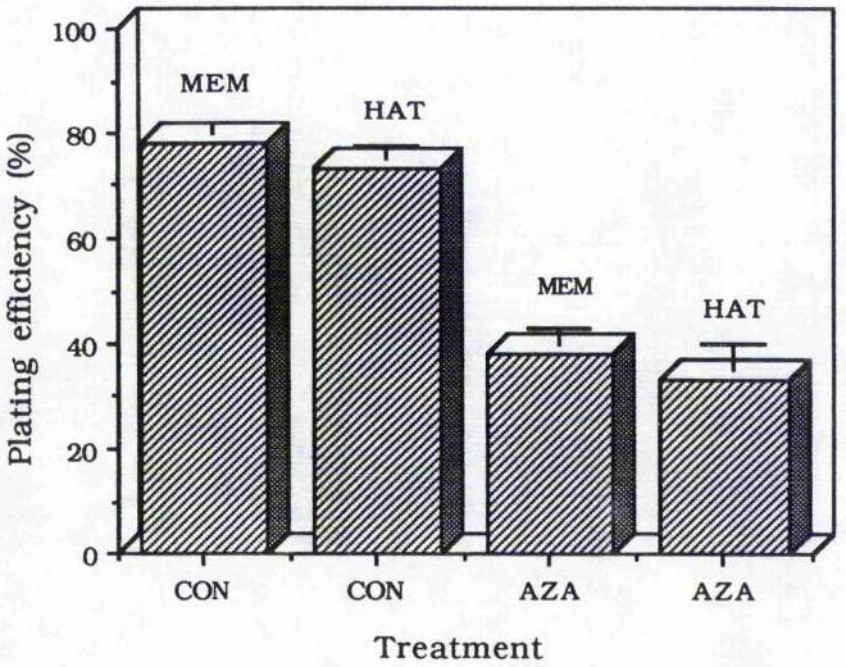


Figure 3.11. The clonal assay following the treatment of CHO KI cells with either no treatment (control) or exposed to 5-azacytadine. Cells are plated in both normal medium (MEM/FCS) and HAT-supplemented medium.

The untreated control samples have nearly the same plating efficiency both in the normal and HAT/MEM medium. In contrast to the mutant cells (figure 3.10), the normal cells treated with AZ show a comparable P.E. when plated in both normal and HAT/MEM medium. However, the AZ treated cells show a reduced P.E compared to the control samples due to the toxic effects of the drug which reduced the viability of the cell population. This result further validates the use of the HAT/MEM medium for the selection of *tk*+ cells.

3.4 Discussion

Due to the limited mutation data based on the use of the CHO/*tk* mutation assay, it was important to establish the three most important parameters which are involved in any mutation assay namely: expression time, concentration of selective agent and the cell density in the selection medium (Cox and Masson, 1976., Chu and Malling, 1968). The mutation versus expression time graph obtained shows a typical relationship with an initial increase, optimum and finally a decrease in mutation frequency (Fig. 3.1). This result is consistent with the results observed by other workers (Chu and Malling, 1968., Bridges and Huckle, 1970). Determination of the optimum expression time is important due to the its dependence on the target locus, cell line and laboratory conditions under which the mutation assay is performed (Thacker et al, 1976., Vrieling et al, 1985). In the CHO/*tk* mutation assay, an expression time of four days was found to yield the maximum induced mutation frequencies independent of the dose (Fig. 3.1). Since the expression time defines the number of cell divisions after irradiation required for the production of a mutant cell, a dose-independent expression time would suggest that mutants induced after exposure to a higher dose (6 Gy) are not at any selective disadvantage during the selection period in comparison to cells exposed to a lower dose e.g. 2 Gy. The dose independence of the expression time at the *tk* locus could be due to the autosomal nature of the *tk* locus (2 copies) which would allow near-optimum growth of the mutant cells despite genetic damage to one of the loci, since the mutant cells would survive due to the undamaged genes (for survival) on the homologous chromosome. This observation is supported by mutation data from mouse lymphoma cells (Evans et al, 1986, 1990) in which the increased ability to recover *tk*- mutants in comparison to *hprt*- mutants has been suggested to be due to the presence of two copies of linked essential genes in the *tk* locus, hence the inactivation of one copy by a lesion affecting the target locus would not necessarily give rise to cell lethality (Evans et al, 1986., DeMarini et al, 1989).

This dose-independent expression time in the CHO/*tk* mutation assay is a distinct advantage which would reduce any artefact in the results obtained since a similar experimental schedule is maintained for all the different doses used. The four days expression time used for the present experiments represented the average time for most mutation assays (Bridges and Huckle,1970). Furthermore, after 4 days most of the treated cell cultures were found to be near-confluent. This avoided the need to trypsinise cells during the expression time (to maintain exponential growth), hence reducing further errors which might arise due to excessive handling of cultures. This also reduces the cost of the assay due to reduced amount of medium which would otherwise be used during the passaging of the cultures.

The effect of increasing TFT concentration on the growth of the wild type cell population is shown figure 3.2. A TFT concentration of greater than 1μg/ml is found to effectively eliminate the background growth of the wild type cell population. A plateau region is observed between 1-30μg/ml, a result similar to that observed by Adair and Carver (1979). Based on this observation, a TFT concentration of 3μg/ml was used to represent the optimum concentration required to select for *tk*- mutant cells. This concentration was chosen to balance between the elimination of growth of the wild type cells and reduce toxic effects of the drug observed at higher concentrations (30 μg/ml). Furthemore, use of this concentration (3μg/ml) was found to give rise to discrete TFT^r colonies which allows an accurate calculation of the induced mutation frequency in a sample. The concentration of TFT used to select *tk*- mutant cells is consistent with results from previous studies (Adair and Carver, 1979., Kronenberg and Little, 1989., Moore et al, 1985a,b).

Some of the early work (Chu and Malling, 1968) suggested the importance of cell density in the selection medium on the yield/recovery of the mutant cells. This variable was investigated for the CHO/*tk* mutation assay via a reconstruction experiment using a prototype *tk*- mutant designated as TK4 (see section 2.7). The optimum cell density was determined by plating a fixed number of TK4 cells plated in the presence of increasing concentrations of CHO KI cells in TFT/MEM to determine the cell density which allowed the maximum recovery of the mutant cells (Fig. 3.3). Based on the results

obtained, a standard cell concentration range of 10^5-10^6 cells per 100 mm dish was maintained in all the experiments described in this thesis. This concentration was adjusted within the range (10^5-10^6) for different experiments to take into account both the radiosensitivity of the cell line and type of mutagenic treatments. This range of cell density has been in a number of mutation assays in various cell lines (Adair and Carver, 1979., Evans et al, 1986, 1990).

Having established the optimum conditions, it was important to confirm the loss of the enzyme thymidine kinase in the persumed *tk*- mutant clones. The presence of the enzyme thymidine kinase is not necessary for cell survival, the cell relying on the *de novo* synthesis of thymidine monophosphate by the enzyme thymidylate synthetase. Thymidine kinase catalyses one of the several 'salvage' pathways in the cell (Kit et al, 1963), enabling the cells to incorporate extracellular source of thymidine. The complete loss of any uptake of ^{3}HTdR in TK4 mutant cells in comparison to the wild type cells measured during DNA synthesis (Fig. 3.4) provides strong evidence for a genetic change at the *tk* locus. This would result in either the complete loss or the production of an altered form of the thymidine kinase rendering it inactive. Further evidence for the loss of thymidine kinase activity in *tk*- mutant cells is provided by the lack of uptake of ^{3}HTdR using autoradiography (Table 3.3, Plate 3.1, plate 3.2). The complete loss of ^{3}HTdR incorporation in the mutant cells provides further support to for the presence of true *tk*- mutants with the complete loss of enzyme activity since a mutant cell population with a reduced/altered form of thymidine kinase would give rise to a few radioactively labelled cells.

Previous work has found some mutant cells to revert back to the normal phenotype when maintained in continuous culture while some cells has been found to be relatively stable. The inability of the mutant cells to revert back to their normal parent phenotype has been used as evidence for a stable genetic change (Chu, 1971., Gillen et al, 1978., Fox et al, 1976). The complete lack of any spontaneous reversion of *tk*- mutant cells (Table 3.2) suggests that the conditions used in the CHO/*tk* mutation assay allow the induction and selection of true *tk*- mutants with a stable change in the *tk* gene, affecting the expression of the gene. This is further supported by the stability of the *tk*- mutant cells in TFT/MEM (colony assay) after an average of about 25 passages

in normal medium to allow for any reversion back to the *tk*+ phenotype (Fig. 3.8). To test the validity of the HAT selection medium for revertants, both the TK4 mutant and parent CHO KI cell line were grown in the presence of both MEM/FCS and TFT/MEM (Fig. 3.5, Fig. 3.6). Both the normal and mutant cell lines grew in the presence of the normal media, further evidence for the inessential property of the *tk* gene. In contrast, the TK4 mutant cells show an increase in cell number compared to the complete loss of growth of CHO KI cells in the presence of TFT/MEM. This result confirms both the use of TFT as an effective *tk*- mutant selective drug and the stability of mutant cells in the presence of continuous selection pressure.

The decreased survival observed in TK4 mutant cells exposed to X-rays in comparison to the parent CHO KI cell line (fig. 3.9) suggests that mutations at the *tk* locus extend to flanking genes which are important for the immediate cell survival after irradiation. This notion is supported by already existing evidence for the predominance of large genomic deletions in cells exposed to ionising radiation (Cox and Masson, 1978., Kavathas et al, 1980., Vrieling et al, 1985., Breimer et al, 1986., Fuscoe et al, 1986). This view is also supported by the observations which suggest that ionising radiation gives rise to multilocus lesions (Evans et al, 1986, 1990) which sometimes extend beyond the target gene effecting and affecting essential flanking genes (Kronenberg and Little, 1988).

Isolation of recessive mutations in autosomal genes has been difficult (Clive et al, 1972) and mutations are recovered at a very low frequency in comparison to mutations on X-chromosome located genes (DeMars and Held, 1972). It has been suggested that this difficulty may to be due to the diploid complement of autosomal alleles in somatic cells (Kit et al, 1963., Kao and Puck, 1968., Chu and Ho, 1970., Clive et al, 1972). Hence the high mutation frequency and the ease of isolation of *tk*- mutants in the present study is contrary to previous studies (Clive et al, 1972., Roufa et al, 1973). This suggests that the CHO KI cell line in our laboratory is hetereozygous at the *tk* locus which is an important property to develop in any mutation assay (Fox, 1971). Three possible genomic stuctures of the *tk* alelle in the hamster cells used for the present studies are presented in figure 3.12, to explain the high mutation frequency observed at this locus.

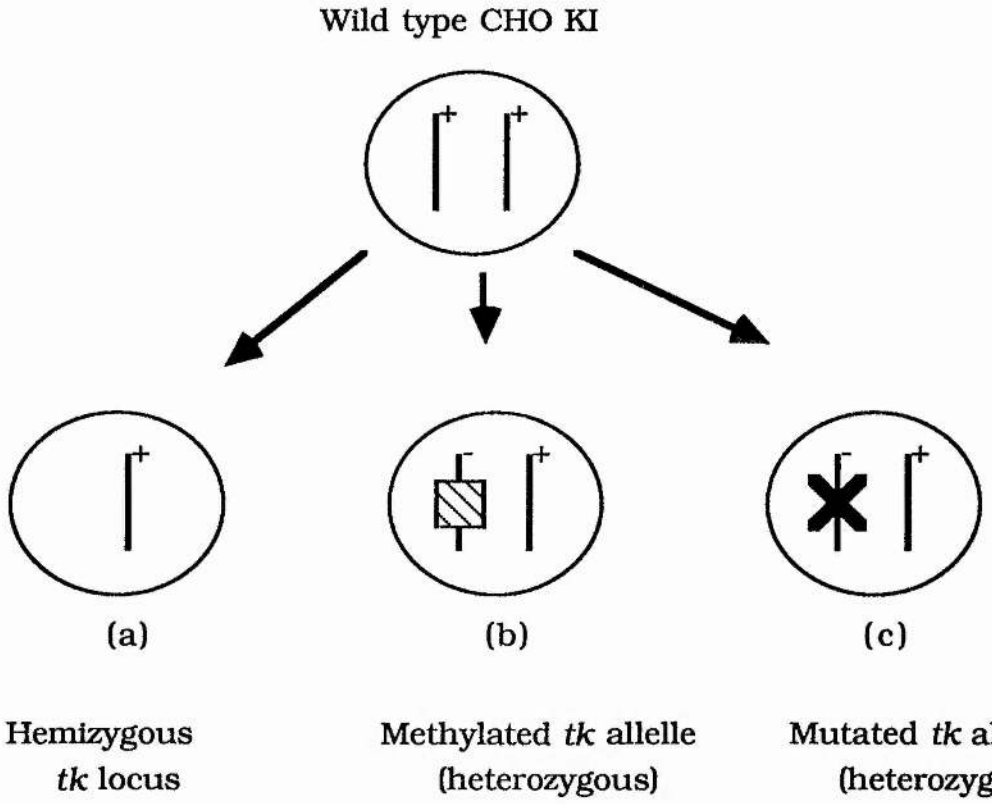


Figure 3.12. Possible genetic state of the *tk* alleles in hamster cells which would allow the isolation of stable *tk*- mutants.

There is increasing evidence which suggests that CHO lines contain a significant hetero- or hemizygosity for a number of autosomal loci (Siminovitch, 1976). However, the precise reason for this deviation from its diploid nature remains unclear. Hence, a possible hemizygous *tk* allele would result in a target locus similar to the *hprt*, thus allowing the easy isolation of *tk*- mutant cells. Such a change has been observed at the autosomally located *aprt* locus in CHO cells in which a number of *aprt*- mutant have been successfully isolated using one or two step mutation assay inspite of the diploid nature of this gene in CHO cells (Meuth and Arrand, 1982., Nalbantoglu et al, 1983) A hemizygous *tk* locus in the CHO cells used is unlikely due to the increased induced mutation frequencies measured at the *tk locus*

compared to those measured at the *hprt* locus (Darroudi and Natarajan in Chinese hamster ovary cells following irradiation. The higher induction of mutations observed at the *tk* locus compared to the *hprt* locus is contrary to the 'target theory'. The *tk* locus (15 Kbp) is smaller in size compared to the *hprt* locus (54 Kbp). Hence, the high mutation frequency at the *tk* locus would suggest the presence of a homologous chromosome which would allow the increased recovery of the mutant cells.

The other two possible *tk* gene structures to explain the ease with which *tk*- mutants can be isolated in the CHO KI cells include the presence of a heterozygous *tk* locus as a result of a mutation (point/deletion) or methylation of one of the *tk* alleles. Such a functional and non-functional autosomal *tk* allelle has been observed in a near diploid human B-lymphoblastoid line (Levy et al, 1968., Liber and Thilley, 1982., Skopek et al, 1978) and in the mouse lymphoma L5178Y cell line (Clive et al, 1979., Moore et al, 1985 a,b). The methylation of one of the *tk* alleles is termed an 'epigenetic' change which may be responsible for the higher than expected frequency of recessive mutants in a number of wild type cell lines due to the hetreozygosity of the locus (Siminovitch, 1976., Jeggo and Holliday, 1986). Such a methylation at one of the *tk* alleles would result in a functional hemizygote (Lieberman et al, 1983), a state which has been shown to give rise to unstable mutations. Using Chinese hamster cells, Morgan and Harris (1982) suggested that the isolated *tk*- mutants were the result of a stable shift in gene expression rather than a mutation in the *tk* locus. This is based on work which suggested that gene activity was inversely correlated with the extent of DNA methylation (Doerfler, 1983., Naveh-Many and Cedra, 1981). Reversion back to a tk^+ phenotype as a result of hypomethylation has been shown to occur in a number of hamster cell lines (Harris, 1982). However in the present study, the *tk*- mutants failed to revert back to a tk^+ competency following treatment with the demethylating agent 5-azacytadine (Fig. 3.10). A similar result has also been observed in *tk*- mutants isolated in the L5178Y mouse lymphoma cell line (Moore et al, 1988). Based on the absence of any revertant tk^- mutants, the possibility of a suppressed methylated *tk* allelle can be eliminated, which further supports evidence against the isolation of unstable or

transient *tk*- mutant cells. Due to the lack of a probe for the genomic *tk* gene, it was not possible to directly confirm the presence of a functional hemizygous or heterozygous *tk* allele. However, having eliminated the possibility of a methylated *tk* allele which would give rise to unstable *tk*- mutants, it can be concluded that the CHO/*tk* mutation assay selects stable genetically altered *tk*- mutants.

CHAPTER 4

Mutation induction by X-rays in CHO KI and *xrs* 5 cell lines

4.1. Introduction

In order to investigate into the effect of defective (or reduced) repair of dsb on the mutation induction, the *xrs* 5 mutant cell line was used. This mutant line was used due to its inherent defective dsb repair ability (Kemp et al, 1984., Costa and Bryant, 1988) while showing a normal ability to repair ssb (Kemp et al, 1984). In this chapter, the mutation induction at the autosomally located *tk* locus is compared between the *xrs* 5 mutant cell line and in its wild type parent cell line CHO KI following exposure to X-rays. The experiments and results described in this chapter throw further light on the the relationship between dsb and mutation induction.

4.1.1 *X-ray-sensitive mutant line (xrs 5)*

Following a number of mutagenic treatements with EMS, Jeggo and Kemp (1983) isolated a number of X-ray-sensitive mutants of the parent CHO KI cell line. Two subgroups of these mutants were isolated which consisted of 6 extremely and 6 moderately radiosensitive lines. The radiosensitive lines placed in a single complementation group (Jeggo, 1985) exhibited a 6-10 fold lower D_{10} value compared to that observed in the parent CHO KI cell line. The *xrs* mutant cell lines have also been shown to be extremely sensitive to other DNA damaging agents. Kemp et al (1984) using neutral filter elution technique related the observed radiation sensitivity in the *xrs* mutants to a reduced ability to rejoin induced dsb. The importance of the *xrs* mutant cell lines is further enhanced by the fact that it has been shown to have no defect in ssb repair (Kemp et al, 1984) thus allowing a study of the effects of a reduced dsb repair on the various biological endpoints observed in irradiated cells. One of the important endpoints which has been widely studied using the *xrs* 5 cell line is an increased chromosomal sensitivity to X-rays (Kemp and Jeggo, 1984., Bryant et al, 1987., Darroudi and Natarajan, 1987a,b) and restriction endonucleases (Bryant et al, 1987). The reduced repair of dsb in the *xrs* cell lines was thought to arise as a result of methylation of one of the *xrs*$^+$ genes thus giving rise to a CHO KI cell which was functionally hemizygous for the *xrs*$^+$ gene (Jeggo and Holliday, 1986) hence a mutation in the unmethylated *xrs* copy would give rise to the observed

repair defect in the *xrs* mutant cells. This hypothesis was confirmed on the basis of the high reversion frequency (to radioresistance) observed in the *xrs* strains following treatment with the methylase inhibitor, 5-azacytadine (AZ) (Jeggo and Holliday, 1986). This suggests that the radiosensitivity of the *xrs* strains arose due to the methylated copy of the *xrs* gene which is not expressed in the mutant strains (Jeggo and Holliday, 1986). Furthermore, the reversion observed in cells treated with AZ is evidence for a structurally intact methylated copy of the *xrs* gene. In addition, the fact that the extremely radiosensitive 6 isolated mutants lie within a single complementation group further suggests that all have have a near-similar type of mutation.

4.2 Materials and methods

4.2.1 *Cell culture and conditions*

Exponentially growing CHO KI and *xrs* 5 cells cultured as monolayers in 75 cm^2 flasks (Sterlin) were used for the experiment described in this chapter. Both the cell lines were maintained as described in section 2.2. Prior to any mutation experiments, both cell lines were maintained in the presence of HAT/MEM for 2-3 days. This procedure was found to be effective in reducing the spontaneously induced (pre-existing) *tk*- mutant cells. This procedure was imposed on the cultures on a regular basis throughout the course of this project.

4.2.2 *Mutation assay*

There is a slight difference in mutation assay performed for the CHO KI and *xrs* 5 cells due to the increased radiation sensitivity of the latter. This results in a slight variation in the mutation protocol for the cell lines which is therefore described separately.

(a) *CHO KI cells:* Cells were diluted to give approximately 2 x 10^5-2 x 10^6 cell per 75 cm^2 flasks supplemented with 10 ml of MEM/FCS. Flasks were incubated at 37°C for 3 hr (to allow a monolayer formation) prior to exposure to X-rays (2,4 and 6 Gy). Cells were incubated for an additional 4 days expression period and maintained in

exponential growth by passaging near confluent cultures as described in section 2.2.

(b) *xrs* 5 *cells:* Due to the radiosensitivity of the *xrs* 5 cell line, the maximum X-ray dose for the mutation assay was limited to 2 Gy. This relatively low cut-off maximum dose was chosen on the basis of the immediate cell survival of cells exposed to X-rays. A higher dose (>2 Gy) would give rise to a low surviving fraction (< 20 %) creating an artefact in the calculation of the true induced mutation frequency. For this reason, *xrs* 5 cells were seeded at cell densities ranging between 2×10^5-2×10^6 and exposed as monlayers to 0.5, 1, 1.5 and 2 Gy of X-rays. Cells were maintained in exponential growth for an expression period of 4 days. This was found to give a good recovery of *tk*- mutant cells similar to that observed in the parent CHO KI cell line. During the expression period, *xrs* 5 cells were passaged at a slightly higher cell number ($5 \times 10^{5)}$ to take into account the increased radiation induced cell death.

4.2.3 *Cell survival*

Immediately after irradiation, a cell survival assay was performed for both cell lines. In case of the CHO KI cells line, cells were diluted to give approximately 100, 400 and 600 cells per 5 cm petri dish supplemented with 5 ml of MEM/FCS in case of 2, 4 and 6 Gy X-ray treatments respectively. For the *xrs* 5 cell line, cells were diluted to give 100-2000 cells per 5 cm dish for the X-ray dose range of 0-2 Gy. Dishes were incubated in a humidified incubator at 37°C with 5% CO_2 in air for 8 days. After the removal of medium, colonies were fixed and stained as already described in section 2.6.

4.2.4 *X-irradiation*

Both cell lines were exposed as monolayers to X-rays as described in section 2.3.

4.2.5 *Determination of spontaneous induced mutations*

Both the CHO KI and *xrs* 5 cell cultures were passaged for 2-3 days in the presence of HAT/MEM prior to setting up cultures for the

mutation assay. This treatement was found to elininate any pre-existing *tk*- mutant cells hence allowing calculation of the induced mutation frequencies by X-rays much more accurately. To measure the spontaneously induced mutations, both cell lines were trypsinised and diluted to give 10^6 cell per 10 cm petri dishes (Sterlin). Dishes were incubated in an humidified incubator set at 37°C with 5% CO_2 in air for 10 days. A simultaneous viability assay was also carried which involved plating 100-200 cells per 5 cm petri dish in normal MEM medium and for 8 days.

4.3 Results

4.3.1 *Cell survival*

The survival curves of both CHO KI and *xrs* 5 cell lines obtained after exposure to increasing doses of X-rays are presented in figure 4.1.

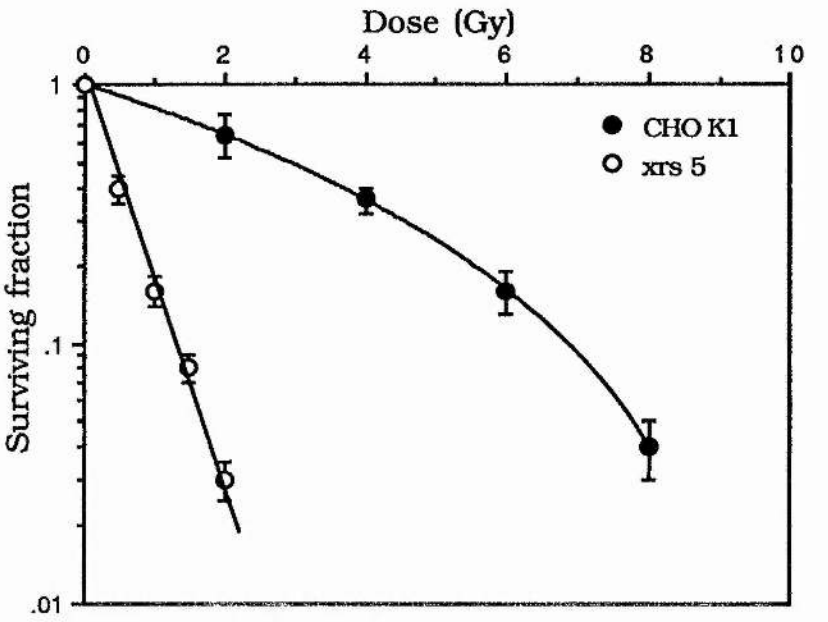


Figure 4.1. Survival curves of X-irradiated CHO KI and *xrs* 5 cells. Vertical bars represent the standard errors of mean values. All the data points shown represent the mean of three independent experiments.

The increased radiosensitivity observed in the *xrs* 5 cell line confirms previous work (Jeggo and Kemp, 1983). The exponential decrease in *xrs* 5 survival is suggested to reflect its inherent reduced ability to repair dsb (Kemp et al, 1984). This result was used to confirm the radiosensitivity of the *xrs* 5 cell line in our laboratory before performing the mutation experiments. This check is particularly important since although Jeggo and Kemp (1983) found the isolated *xrs* mutant lines to be stable with regard to the phenotype, it was later reported that some strains (*xrs* 6) revert back to a level of radioresistance similar to that of the wild-type cell line (Denekamp et al, 1989).

4.3.2 *Spontaneosly induced mutation frequencies*

The results of the spontaneously induced mutation frequencies assayed following HAT treatment are presented in table 4.1.

Cell type	Spontaneous mutations (per 10^5 survivors)	Standard error of mean
CHO KI	3.64	2.6
xrs 5	10.6	2.5

Table 4.1. Spontaneous-induced mutation frequencies in both the CHO KI and *xrs* 5 cell lines. All the values indicated are the average of four independent experiments.

The *xrs* 5 cell line shows a near 3-fold increased spontaneously-induced mutations compared to that observed in the parent CHO KI cell line. A similar enhanced background mutation frequency has also been observed in the *xrs* 5 cell line measured at the *hprt* locus (Darroudi and Natarajan, 1989). The spontaneously induced mutation frequencies are between the acceptable range (< 20 mutations per 10^5 survivors) which is important in reducing the chance of obtaining any false negative results for weak mutagens due to masking by the high

background (Nestmann et al, 1991). All the data shown above represents the mean of at least four indepenedent experiments.

4.3.3 *CHO KI and xrs* 5 *mutation induction curves*

The results of the mutation experiments performed for both the CHO KI and *xrs* 5 cell lines exposed to increasing doses of X-rays are presented in figure 4.2.

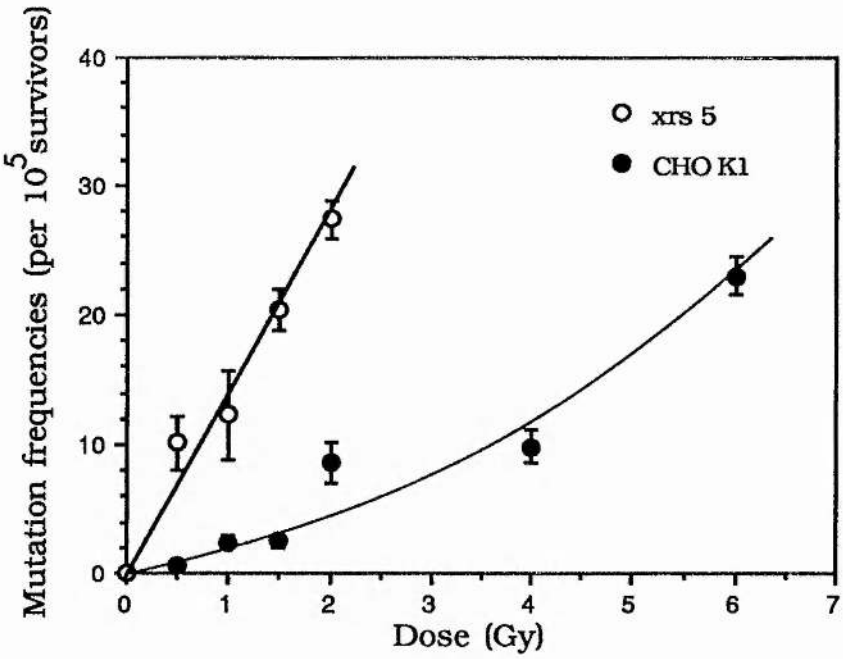


Figure 4.2. Plot of mutation frequencies as a function of X-ray dose for both CHO KI and *xrs* 5 cell lines. Vertical bars represent the standard errors of mean values. Each curve represents the mean of three independent experiments.

The frequency of mutations in CHO KI cells showed a curvilinear response with increasing X-ray dose while the *xrs* 5 data approximated to a linear increase in mutation frequency with increasing dose. The frequency of mutations per 10^5 survivors was 3-4 times higher in the *xrs* 5 than in the parental CHO KI cell line, the ratio of mutation frequency between the two cell lines varying with dose. The hypermutability observed in *xrs* 5 cells is consistent with

earlier reports (Darroudi and Natarajan, 1989., Tesmer et al, 1986) in which the mutations were measured at the *hprt* locus. The average spontaneous mutation frequencies of 3.64 and 10.6 per 10^5 viable cells in CHO KI and *xrs* 5 cells respectively (Table 4.1) have been subtracted from the data points shown which represent the mean of three independent experiments.

4.3.4 *5-azacytadine treatment*

In the previous chapter, the *tk*- mutants isolated from CHO KI cells were shown to be stable following treatment with AZ (section 3.3.10). Similarly, a *xrs* 5 *tk*- mutant designated as *xrs* (*tk*-), the isolation of which is descibed in section 2.6 was also tested for its stability following treatment with AZ. The results of the colony assay following treatment of the *xrs*(*tk*-) mutant cells with AZ are presented in figure 4.3.

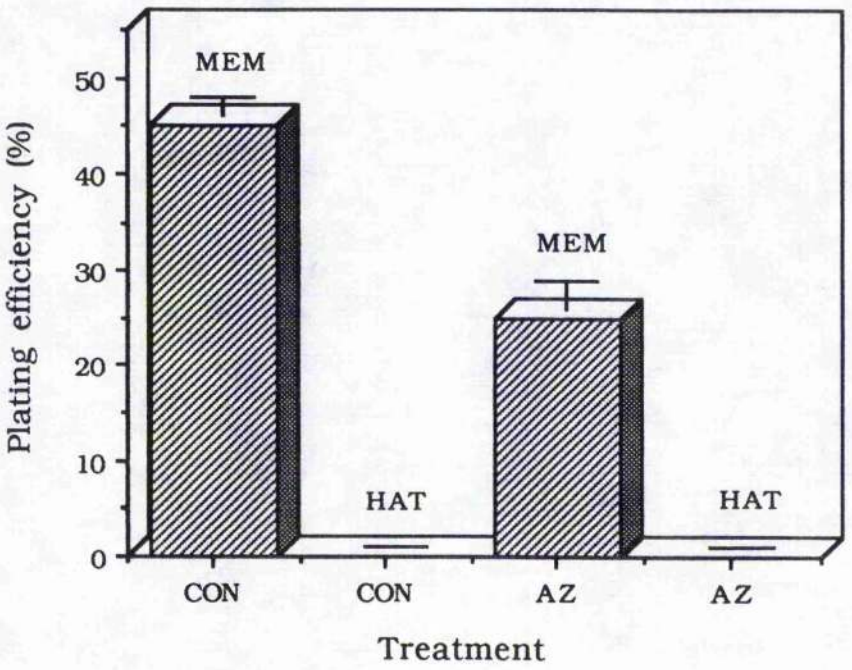


Figure 4.3. Plating efficiency of *xrs* (*tk*-) mutant cells in both HAT-supplemented and normal MEM medium following treatment with AZ. The plating efficiency of the untreated cell population in normal medium is also shown (con).

A complete loss of cell survival is observed in dishes supplemented with HAT/MEM following treatment of the mutant line with AZ and also in case of untreated (control) cell populations. This result eliminates the possibility of an epigenetic change at the *tk* locus resulting in the *xrs* (*tk*-) mutant clone. This is result is similar to that observed in case of the CHO KI mutant line (section 3.3.10) which would suggest that both *xrs* 5 and CHO KI cell lines undergo near similar stable genetic changes at the *tk* locus to give to the resulting *tk*- mutant cells.

4.3.5 *DNA synthesis*

Both *xrs* 5 and *xrs* (*tk*-) cells were incubated in the presence of ^{3}HTdR and measured uptake of activity via the salvage pathway used as evidence for the presence/absence of cellular thymidine kinase. The amount of ^{3}HTdR activity incorporated into the DNA (measured in terms of dpm in precipitated and TCA washed DNA) at the various sampling times is shown in figure 4.4.

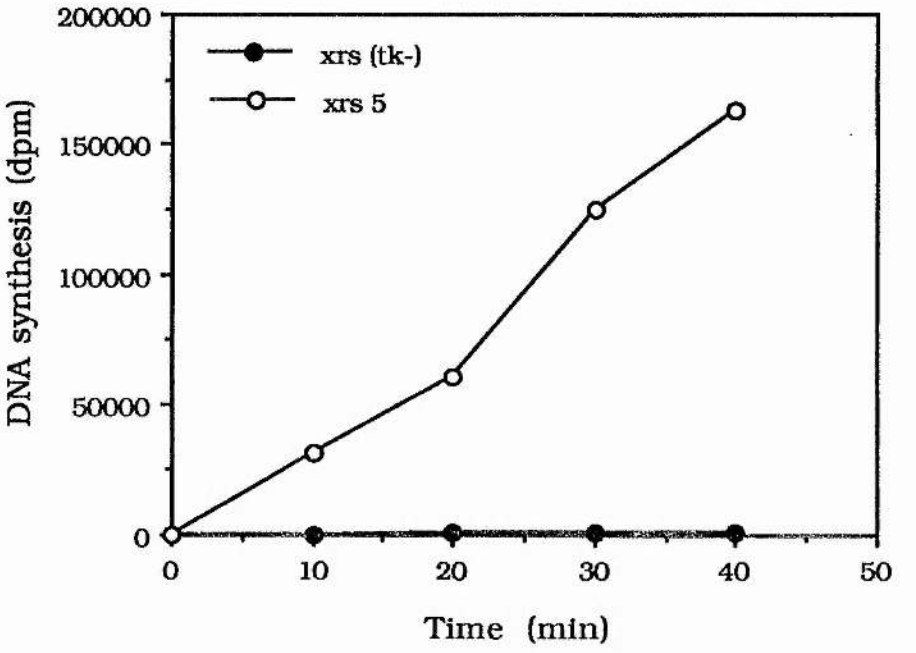


Figure 4.4. Uptake as a function of time of ^{3}HTdr (dpm) measured in the *xrs* (*tk*-) mutant and *xrs* 5 wild type cell population following incubation at 37°C.

The *xrs* 5 cells show a linear increase in the incorporation of ^{3}HTdR in comparison to the *xrs*(*tk*-) cells which show a complete loss of uptake of radioactivity measured at different sampling times.

4.3.6 *Comparison of tk and hprt induced mutations.*
A comparison of the X-ray induced mutations in both the CHO KI and *xrs* 5 cell lines measured at the *tk* and *hprt* loci are presented in figure 4.5.

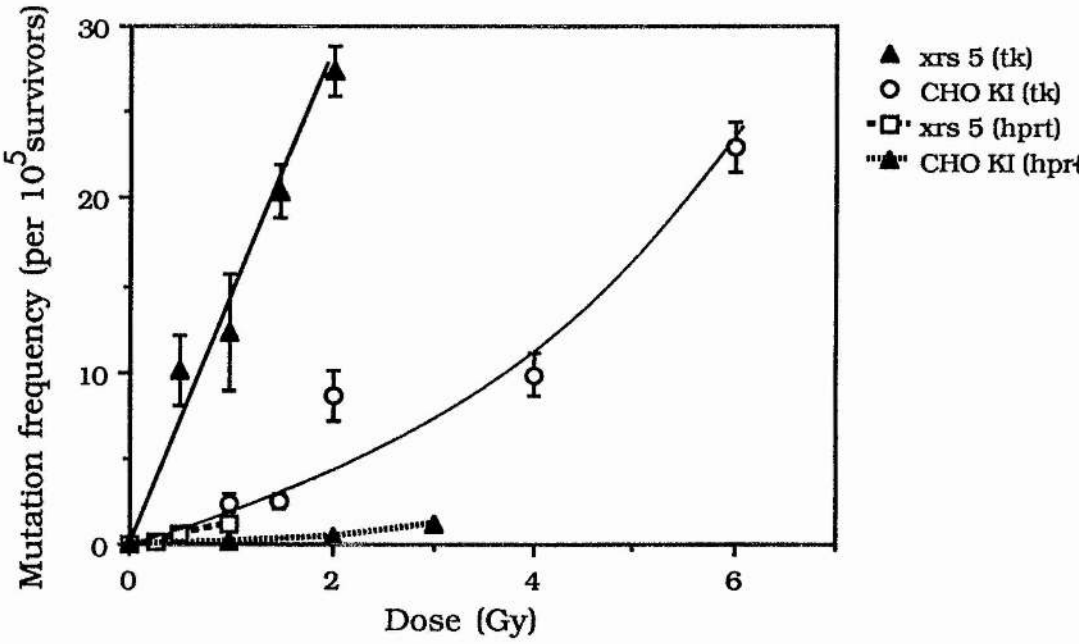


Figure 4.5. Comparsion of the induced mutation frequencies induced following treatment of both the *xrs* 5 and CHO KI cell line measured at the *tk* and *hprt* loci. The *hprt* mutation data has been redrawn from Darroudi and Natarajan, 1989. Vertical bars (in case of the *tk* loci data) represents the standard errors of mean values from three independent experiments.

The data of *hprt* induced mutations has been replotted from Darroudi and Natarajan (1989) for the purpose of comparison. Mutations measured at both the *tk* and *hprt* (Darroudi and Natarajan,

1989) loci in the *xrs* 5 cell line shows an enhanced mutational response compared to the CHO KI induced mutations. However, the *tk* locus shows an increased sensitivity to X-ray induced mutations (factor of 5-10) compared to those measured at the *hprt* locus. This increased mutability at *tk* in comparison to *hprt* loci has been observed in other cell lines (Evans et al, 1986., Stankowski and Hsie, 1986., Yandell et al, 1986., Moore et al, 1987).

4.3.7 *Relationship between mutation and survival*

A plot of induced mutation frequency versus log surviving fraction for both CHO KI and *xrs* 5 cells is presented in figure 4.6.

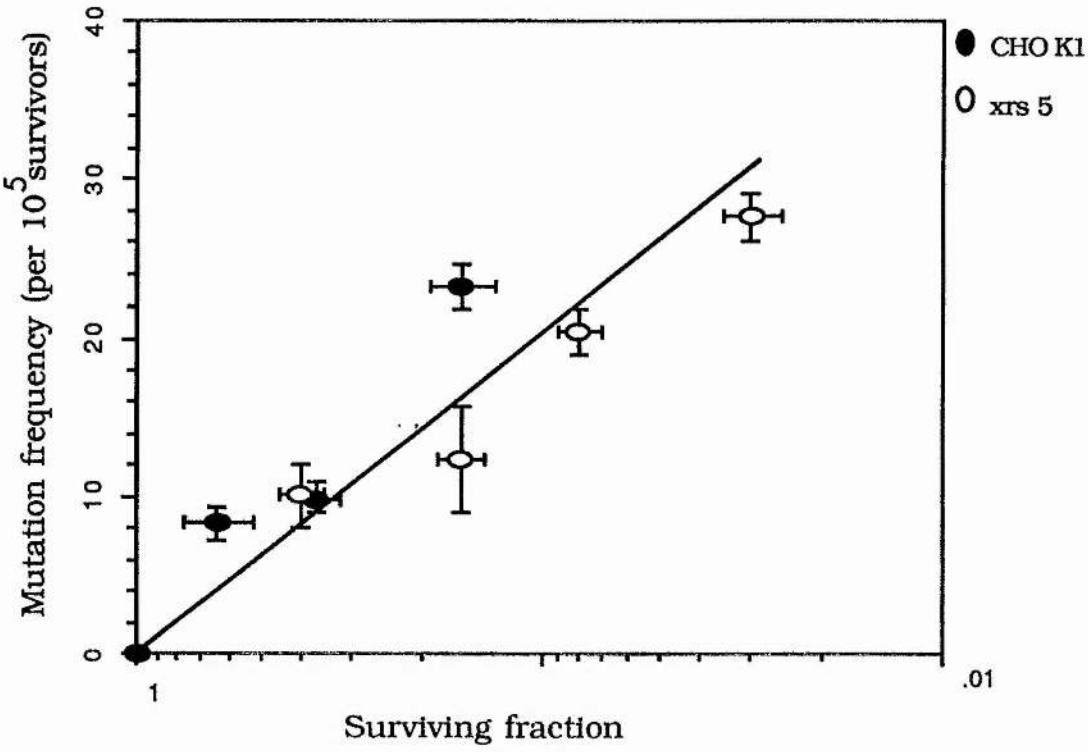


Figure 4.6. Plot of induced mutation frequency versus surviving fraction from both CHO KI and *xrs* 5 cells following treatment with X-rays. Both vertical and horizontal bars represent the standard errors of mean values from three independent experiments.

Both sets of data show an increase in mutation frequency with a decrease in cells survival. A common line can be approximately be fitted to both sets of data i.e CHO KI and *xrs* 5 cells. Such a linear relationship has been reported by other workers measured in different cell lines (Munson and Goodhead, 1976., Chadwick and Leehouts, 1976, Thacker et al, 1977., Thacker and Cox, 1975) which has been interpreted as an indication for a constant relationship between radiation-induced mutations and cell inactivation (Thacker and Cox, 1975).

4.4 DISCUSSION

The large number of radiation-induced products induced in the DNA following treatment with ionising radiation (Teoule and Cadet, 1978., Hutchinson, 1985) has made the task of correlating radiation mutagenesis to a specific lesion very difficult. It is not only important to identify a specific lesion which gives rise to a mutation to have a better understanding of the genetic lesions responsible for the various types of human genetic disorders but the resolution of these lesions would also lead to a better understanding into the various molecular processes involved during the process of mutagenesis e.g. repair, reproductive lag and its final metabolic consequences. With the isolation of various mutant cell lines known to be defective in the repair of specific lesions (Thompson et al, 1982., Kemp et al, 1984), a new window has opened into elucidating and understanding the various mechanisms involved in a number of biological processes. More recently, with the isolation of radiosensitive mammalian mutant cell lines (Jeggo and Kemp, 1983., Zdzienicka and Simons, 1987., Jones et al, 1987) which can be maintained in culture, a lot of work has been developed into a better understanding of radiation-induced mutagenesis in addition to other biological endpoints. Base alterations have been shown to give rise to mutations in bacteria, bacteriophages and lower eukaryotes (Glickman et al, 1980., Malling and deSerres, 1973) but the role of this type of damage in radiation mutagenesis in mammalian cells remains unclear (Arlett et al, 1975). Hence, the present study was directed at investigating into the role of the predominant type of damage produced in cells exposed to ionising radiation, namely strand breakage on mutation induction. Both ssb and dsb constitute the two major types of DNA strand breaks both of which are thought to play an important role in both cytogenetic and cytotoxic responses in irradiated cells. However, there is increasing evidence indicating dsb to be the more important lesion responsible for the formation of various radiation-induced end-points which include chromosomal aberration formation, oncogenic transformation and cell death (Bryant, 1988, review).

Based on the putative importance of dsb, this study investigated the role of dsb on the induction of radiation-induced mutations measured at the *tk* locus in both the parent CHO KI and its

radiosensitive mutant *xrs* 5 cell line. This radiosensitive mutant line isolated by Jeggo and Kemp (1983) is known to be defective in rejoining of dsb (Kemp et al, 1984., Weibezahn et al, 1985., Costa and Bryant, 1988) but not in repair of ssb (Kemp et al, 1984) which allowed the study specifically into the effect of defective repair and consequent unrepaired dsb on mutation induction. As a first step in the experiments performed, the hypersensitivity to X-rays of the *xrs* 5 cells maintained and used in our laboratory was checked and confirmed by the results of the survival assay presented in figure 4.1. The increased radiosensitivity observed in the *xrs* 5 cell line evident with an exponential decrease in survival with the lack of the shoulder region confirms previous work (Jeggo and Kemp, 1983). This survival assay was important to perform prior to doing any mutation studies as an important check of any possible reversion of *xrs* 5 cells maintained in continous culture to a level of radioresistance similar to that of the wild type CHO KI cell line (Costa, 1990).

The *xrs* 5 cell line showed a 3-4 fold enhanced mutational response compared to the parent CHO KI cell line following treatment with X-rays, the ratio of mutation induction between the two cell lines varying with increasing X-ray dose (Fig. 4.2). Since dsb is the principal lesion which is left unrepaired in larger numbers in the *xrs* 5 cells than in CHO KI cells, the observed hypermutability provides obvious support for the involvement of dsb as a important pre-mutagenic lesion in X-ray induced mutagenesis. This view is supported by earlier reports (Darroudi and Natarajan, 1989) who also observed an increased induction of mutations in *xrs* 5 cells (2-3 times) compared to those observed in the parent CHO KI cells following treatment with X-rays, mutations in this case measured at the hemizygous *hprt* locus. Further evidence for the importance of dsb as a pre-mutational lesion is provided by the high efficiency of mutation induction observed in cells exposed to cellular ^{125}I-decay, a radioisotope that predominantly produces dsb (Krisch and Sauri, 1975).

Molecular analysis of thymidine kinase deficient mutants induced in various cell lines following exposure to ionising radiation show a large percentage to have undergone alterations ranging from large changes to the complete loss of the *tk* allele (Kronenberg and Little, 1989., Evans et al, 1986, 1990). Due to the unavailibility of the cDNA probe for the genomic hamster *tk* gene in the present study, it

was not possible to check the extent of gene damage in the *tk*-mutants isolated. However, it would not be unreasonable to suggest that majority of the mutations at the *tk* locus in the present study are likely to be large deletions with a lower frequency of other minor changes (base damage) based on the overwhelming evidence suggesting major structural alterations at the DNA level in radiation-induced mutants analysed at other different loci (Kavathas et al, 1980., Orr et al, 1982., Vrieling et al, 1985., Thacker, 1986., Liber et al, 1987., Yandell et al, 1986., Stankowski and Hsie, 1986., Breimer et al, 1986., Graf and Chasin, 1982). This view is supported by the increased proportion of deletion mutants observed in the radiosensitive L517Y-S1 cell line (Evans et al, 1986., 1990) which has been shown to be deficient in dsb repair (Wlodek and Hittleman, 1987). Additional evidence supporting the causal relationship between dsb and deletion mutants is provided by the spectrum of mutations induced in cells exposed to a decay of ^{125}I, the majority of which are found to have deletions ranging from a few base pairs to large deletions of about 20-30 Kbp (Martin and Haseltine, 1981., Martin and Holmes, 1983., Gibbs et al, 1987). This observation is further supported by the similarity in the spectrum of ^{125}I decay and X-ray induced mutations verified following southern blotting analysis of the mutant cell DNA (Gibbs et al, 1987).

The next question is: how does a small lesion such as a dsb give rise to the hypermutability observed in cells in which dsb-repair is inhibited. The role of misrepair of induced damage in increasing the yield of radiation-induced mutations was reported in some earlier observations (Speyer, 1965) which were suggested to arise due to the presence of a mutant DNA polymerase thus affecting the replication repair of the induced damage. Further evidence was provided by Hastings et al, (1976) who based on a study with radiation-sensitive mutants of yeast cells, implicated the observed hypermutability to an increased error-prone repair. More recent experiments have suggested the existence of a correlation between the ability of the cells to recover from sub-lethal damage and the ability to repair pre-mutational damage (Suzuki and Okada, 1977., Nakamura and Okada, 1981). This report is substantiated by a study using cell lines with various radiosensitivities in which a decrease in mutability was observed with an increasing shoulder region in the survival curves

(Fox, 1974). All these suggest the importance of the repair/misrepair process in the resulting induction of a mutations. Repair of dsb in many mammalian cells has been thought to occur via a recombination process (Resnick, 1976) in both lower (Szostak et al, 1983) and higher eukaryotes (Kucherlapati et al, 1984). Dsb have been suggested to stimulate recombinogenic-type repair which if unsuccessful would lead to the formation of large deletions (Gibbs et al, 1987). Although some repair deficient or radiation sensitive cell lines are found to be more mutable than their parental radio-resistant strains (Darroudi and Natarajan, 1989., Evans et al, 1986), a notable exception is the AT cells which although more sensitive than the normal cells, a hypomutability has been reported (Arlett and Harcourt, 1978). From an analysis of DNA strand breaks, AT cells have been shown to be proficient in dsb repair (Lehmann and Stevens, 1977) although observed at a lower fidelity rate (Cox et al, 1986). The lack of an error-prone repair process in AT cell has been suggested as one of the possible factors responsible for the observed hypomutability (Arlett and Harcourt, 1983). This supports evidence for the importance of the error-prone repair pathway in mutation-induction. In view of the high mutability observed in case of *xrs* 5 cells, it could be suggested that in addition to misrepair of dsb, an increased probability of interaction of dsb held open in *xrs* 5 cells resulting in fixation of the damage would account for the hypermutability. This view is supported by studies in which a reduced mutation frequency is observed in cells exposed to a fractionated dose of radiation which is thought to reduce the probability of lesion misrepair (Asquith, 1977)

The *xrs* (*tk*-) mutant cells isolated following exposure to 1 Gy of X-rays were used to confirm the loss of thymidine kinase activity which supports the presence of a genetic change at the *tk* locus. The spontaneous reversion frequency per survivor of less than 10^{-6} in *xrs*(*tk*-) cells support the presence of a stable genetic change at the *tk* locus. This view is further supported by the results of the DNA synthesis assay (Figure 4.4) in which the mutant cells show no uptake of activity (^{3}HTdR) in comparison to the linear uptake of activity observed in the *xrs* 5 (non-mutagenised) cell population. A methylated copy of the otherwise functional *xrs* repair gene in the *xrs* strains has been shown to allow the increased radiation sensitivity observed in these cells (Jeggo and Holliday, 1986). Treatment with AZ, an

inhibitor of DNA methylation has shown to have increased the surviving fraction in *xrs* cells (Jeggo and Holliday, 1986). Such a phenomenon in mutagenesis has been shown to give rise to epigenetic mutations which are generally unstable thus cannot be classified as true mutants. However *xrs* (*tk*-) cells show no reversion to a *tk*+ phenotype (Figure 4.3) following treatment with AZ which suggest a structural change exists in the other functional *tk* gene rather than a suppression of the *tk* gene activity due to methylation. All these analyses of the *xrs* (*tk*-) mutant cells confirm the lack of *tk* gene activity and furthermore are similar to results obtained with the *tk*- mutant cells obtained after treating CHO KI cells with X-rays (i.e TK4: see chapter 3). This similarity would suggest a similar type of mutation in both CHO KI and *xrs* 5 cells which leads to the *tk*- phenotype.

Mutations measured at the *tk* locus show a nearly 5 fold enhanced mutational response in comparison to the *hprt*-induced mutations in both CHO KI and *xrs* cells treated with X-rays (Figure 4.5). This is in agreement with previous studies which also observed a high ratio of mutant frequencies obtained when the target gene is heterozygous e.g. *tk* (DeMarini et al, 1989., Evans et al, 1986., Waldren et al, 1979., Stankowski and Hsie, 1986). The difference of mutability between the *tk* and *hprt* loci is supported by the suggestion that a class of mutations may be recovered at the heterozygous locus while being lethal at the hemizygous locus. This view is supported by observation of the two size i.e large and small TFT^r mutant colonies recovered in mutation assay in mutation assays using L5178Y cells (Moore et al, 1987., Evans et al, 1986., Yandell et al, 1986., Stankowsi and Hsie, 1986). The small colonies have been shown to have undergone multilocus deletions which affect essential genes linked to the *tk* gene while the large colonies are representative of single-gene mutations (Moore et al, 1985 a,b). The inability of the *hprt* locus to recover deletions affecting neighbouring essential genes is evident from the absence of small-colony *hprt*- mutants in L5178Y cells which is suggested to be due to non-viability of these mutants (Clive et al, 1980). The importance of a homologous chromosome in the recovery of mutant cells was evident from some early experiments by Webber and de Serres (1965) who reported increased lethal effects in haploid strains of *Neurospora Crassa*. Further evidence was provided by mutation experiments using a cell strain (LY-R83) which is known to

have a single chromosome (number 11) carrying the *tk* locus in which the induction of mutations were similar to those observed at the *hprt* locus following treatment with X-rays (Wolff, 1971., Graf and Chasin, 1982., Waldern et al, 1979). Furthermore, the high mutability observed at the *tk* locus than at the *hprt* locus support the suggestions that a different mechanism operates for the generation of mutants at autosomal hetereozygous loci. Based on above mentioned observations, the enhanced mutability in the *xrs* 5 cells (Figure 4.2) despite the increased radiation sensitivity suggests that the mutational events outnumber the lethal events at the *tk* locus possibly due to the presence of active copies of the linked essential genes on the homologous chromosome which thus increases the viability of the mutant cell population recovered following treatment.

Mutation-survival plots are difficult to interpret but have been useful in comparing the mutation-inducing ability of mutagens whose 'dose' is difficult to estimate accurately e.g the amount of chemical mutagen absorbed into cells differs from the calculated concentration hence by relying on the more accurate survival assay, a much more accurate determination of mutation-inducing ability obtained. From the data presented in this chapter, both the *xrs* 5 and CHO KI data can be reasonably be fitted with a common line in a mutation-survival plot (Figure 4.6). Previous authors (Thacker and Cox, 1975) have also observed such a linear relationship to exist between cell lines of different radiosensitivities e.g. human diploid fibroblasts and Chinese hamster V79 cells which is interpreted in terms of a fixed probability of mutation induction relative to the lethal effects of the ionizing radiation (Thacker et al, 1977). Such a linear plot can also be interpreted as evidence for a common type of lesion (dsb?) which allows cell survival but is fixed and expressed as a non-lethal mutation. Hence from the reduced dsb repair of the *xrs* 5 cell line (Kemp et al, 1984), it might be suggested that dsb represent pre-mutational lesions which are fixed as non-lethal mutations in cells treated with X-rays (both CHO KI and *xrs* 5 cells). The increased number of residual dsb in *xrs* 5 cells would increase the probability of both lethal and misrepaired dsb the latter if non-lethal could account for the hypermutability observed in *xrs* 5 cells.

Cytogenetic analysis of *xrs* 5 cells exposed to X-rays show a higher number of chromosome aberrations (exchanges and deletions)

in comparison to those observed in the parent CHO KI cell line (Bryant et al, 1987., Darroudi and Natarajan, 1987a,b., Kemp and Jeggo, 1986). These results, in view of the high mutation induction observed in *xrs* 5 cells, would suggest radiation-induced dsb as a common lesion for both the formation of chromosome aberrations and induction of mutations. This notion is supported with the observation that a proportion of radiation-induced *hprt* mutants both in human diploid fibroblasts (Cox and Masson, 1978) and in Chinese hamster cells (Thacker, 1981., Brown and Thacker, 1984) are associated with cytological detectable X-chromosome changes which carries the *hprt* gene and which includes deletions and translocations.

In conclusion, it can be suggested that in a mechanism similar to that postulated for the formation of chromosome aberrations formation (Bender et al, 1974., Natarajan and Obe, 1984) mutations arise as a result of an misrepair of the induced dsb via the formation of chromosome aberrations in particular deletions or a non-lethal exchanges.

CHAPTER 5

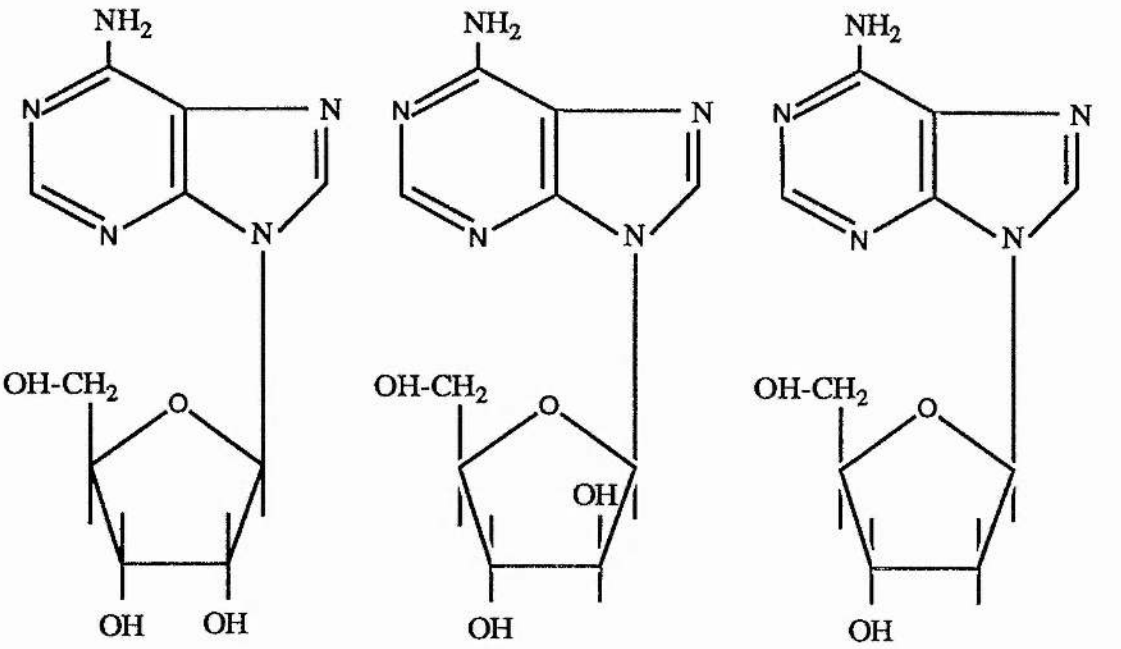
Effect of dsb repair inhibition using ara A on mutation induction

5.1 Introduction

To further investigate the role of radiation-induced dsb in the process of mutagenesis, the effect of inhibition of dsb repair in the parent cell line, CHO KI was investigated with cells exposed to X-rays alone or in combination with ara A and the subsequent induction of mutations measured at the *tk* locus. This work was based on earlier observations of Bryant and Blöcher (1982) and Iliakis and Bryant (1983) who found repair of dsb to be inhibited in the presence of two nucleotide analogues, ß-ara A and ß-ara C in Ehrlich ascites tumour cells (EAT) measured by neutral velocity sedimentation and DNA unwinding techniques.

5.1.1 *Properties and mode of action of ara A*

The drug 9-ß-D-arabinofuranosyladenine (ara A) is a nucleotide analogue of deoxyadenosine which has been shown to have a strong inhibitory effect on DNA synthesis (Doering et al, 1966., Müller, 1979). This drug, first synthesized chemically in 1960 (Lee et al, 1960), has an identical molecular structure to the adenosine except for the 2' position in the sugar moeity. But ara-ATP competes with dATP (deoxyadenosine triphosphate), not ATP (adenosine triphosphate), so although it is more similar in structure to adenosine, it somehow mimics the analogue deoxyadenosine to give rise to the observed DNA synthesis inhibitory action. The chemical structures of the three nucleotides are presented in figure 5.1.



Adenosine Arabinofuranosyladenosine Deoxyadenosine

Figure 5.1 Chemical structure of adeosine and its two analogues, ara A and deoxyadenosine.

In addition to its strong antiviral properties (Shannon, 1975), ara A has also been shown to have important antitumour cytotoxic properties (Ortiz et al, 1972., Gale and Foon, 1986) which have been suggested to be useful in some forms of cancer therapy (Lee et al, 1960) in conjunction with radiation therapy (Iliakis, 1980). Due to its identical structure to the adenosine, this drug has been shown to readily penetrate the cell membrane (Müller et al, 1975., Brink and LePage, 1964) where it acts on DNA-dependent polymerases (Brink and LePage, 1964., Doering et al, 1966., Furth and Cohen, 1968., Müller et al, 1975, 1977., Dicioccio and Srivastava, 1977., Okura and Yoshida, 1978) to inhibit DNA synthesis. Out of the three DNA polymerases in mammalian cells (Miller and Chinault, 1982), ara A has been found to selectively inhibit polymerase α and ß (Furth and Cohen, 1967., Müller et al, 1975., Okura and Yoshida, 1978). Studies on DNA synthesis show that ara A is readily phosphorylated on entry into the cell. The main component of intracellular ara A (60%) is in the form of ß-ara ATP while the rest is present as both mono- and di-phosphates (Müller et al, 1977).

In a study using *Escherichia Coli* (Ohno et al, 1989) and Herpes virus (Müller et al, 1977), the phosphorylated form of ara A was found to be incorporated into the DNA, resulting in chain termination due to inhibition of DNA synthesis. In mammalian cells, ara A has been shown to be incorporated into the DNA only in small amounts hence does not result in any significant chain termination. The phosphorylated form of ara A in mammalian cells is thought to bind to DNA polymerase, competitively inhibiting the utilization of the normal substrate, dATP (Müller et al, 1975). The relative inhibition of α and ß DNA polymerases by ara A has been controversial. Some studies have found inhibition of α polymerase (Müller et al, 1977., Okura and Yoshida, 1978., Stammberger et al, 1989) while others have found an equal inhibition of both α and ß polymerase (Dicioccio and Srivastava, 1977). In a more recent study (Miller and Chinault, 1982), the type of polymerase involved in repair synthesis was suggested to be dependent on the 'patch size' or the extent of damage induced in the DNA. Polymerase ß is proposed as playing a role in 'short-patch' repair (1-5 nucleotides) while polymerase α is thought to be involved in repair involving a 25-50 nucleotide insertion (Miller and Chinault, 1982., Wang and Korn, 1980., Cleaver, 1984). The predominant type of X-ray induced damage is believed to involve the 'short-patch' repair mechanism (Painter and Young, 1982., Fox and Fox, 1973) hence polymerase ß has been suggested as more likely to be involved than polymerase α during inhibition of the repair by ara A (Iliakis et al, 1982., Mirzayans et al, 1988)

5.2. Materials and methods

5.2.1 *Cell culture*

For all experiments the parent CHO KI was used, maintained and passaged as described in section 2.2. However, for all mutation experiments (except for DNA synthesis measurements) with ara A, plateau-phase cell cultures were used. Prior to performing the mutation experiments, cells were passaged in HAT/MEM for 3-5 days to eliminate any pre-existing *tk*- mutant cells.

5.2.2 *Inhibition of DNA sythesis by ara A*

Unlabelled exponentially growing CHO KI cells, at a concentration of 5 x10^5 cells/ml in V-tubes were placed in a waterbath at 37°C and allowed to equilibrate for 15 minutes. Ara A was added in the form of a 45 mM solution in 0.2 M HCl to various final concentrations (25-200 μM). Cells were then incubated for 30 minutes in the presence of ara A to allow the formation and equilibration of a pool of ara ATP. 100 μl (1μCi) of ^{3}HTdR was squirted into each sample at various times. After incubation with ^{3}HTdR for 10 minutes at 37°C, 5 ml of ice-cold saline was added forcefully and samples were placed on ice. When all the samples had been accumulated on ice, they were centrifuged, the supernatant aspirated and the pellet vortexed. 1 ml of 0.03 M NaOH was added to lyse the cells, followed 10 minutes later by 1.5 ml of 0.62 M trichloroacetic acid (TCA). Samples were stored overnight at 4°C to allow precipitation of the DNA. The DNA was then collected onto glass-fibre filters (Whatman), rinsed twice with ice-cold 0.31 M TCA and once with ice-cold ethanol. Filters were transferred to scintillation vials and 4 ml of scintillation cocktail (Optiphase MP, LKB) added. The radioactivity per filter was determined using a liquid scintillation counter (LKB).

5.2.3 *Ara A treatment in mutation experiments*

Approximately 10^5 cells were plated in 25 cm^2 flasks (Sterlin) and grown for 3-4 days to allow the cells to attain the stationary phase of growth. Ara A was added in the form of a 45 mM solution to the stationary cultures to give a final working concentration of 100 μM. The flasks were incubated for 45-60 minutes before irradiation. This pre-irradiation treatment has been found to maximize the effect of ara A (Iliakis, 1980) and is also thought to represent the time required for the formation and equilibration of a pool of ara ATP (Bryant and Blöcher, 1982). Following X irradiation, cells were further incubated for 3 hrs in the presence of ara A before trypsinising and plating at a lower cell density (2 x 10^5 cells) in 75 cm^2 flasks (Sterlin) in fresh normal medium. Cells were incubated for an additional 4 days which was found to be the optimum expression time (section 3.3.1) before plating out for the mutation assay. A simultaneous cell survival assay was carried out after the 3 hrs post-irradiation period. Cells were diluted to give approximately 100-800 cells per dish (depending on

the radiation dose) and plated in MEM/FCS in 5 cm petri dishes (Sterlin). These dishes were then incubated at 37°C for 8 days. The resultant colonies were rinsed twice with Sorenson's buffer (pH 6.4), fixed in methanol for 20 minutes and stained with concentrated Giemsa.

5.2.4 *Mutation assay*

The mutation assay used to measure the induced mutation frequencies in cells exposed to X-rays alone or in combination with ara A, was similar to that already described in section 2.3. The modifications in the experimental protocol are summarised in figure 5.2.

5.2.5 *Ara A dose response*

Stationary cell cultures in 25 cm^2 flasks (Sterlin) were exposed to 4 Gy of X-rays in combination with increasing concentrations of ara A (25-400 μM). Parallel control flasks with similar concentrations of ara A were also set up, but were not exposed to X-rays. Following irradiation, the protocol was similar to that described in section 2.4 to determine the induced mutation frequencies.

5.2.6 *Measurement of chromatid breaks using the G_2 assay*

For each experiment, 10 flasks (75 cm^2) were set up to attain a plateau-phase cell culture (CHO KI) in the presence of MEM/FCS. These included a control sample, ara A control, 4 flasks treated to X-rays alone and 4 flasks exposed with X-rays in combination with ara A (100μM). All the samples were exposed as monolayers to an X-ray dose of 0.75 Gy. To each sample, ara A was added and incubated at 37°C for 60 minutes prior to irradiation. This was followed by 3 hr post-irradiation incubation at 37°C in the presence of ara A. The conditions were kept identical to those maintained for the mutation assay (section 2.4). Following irradiation, cells were harvested at different times to prepare slides. Metaphases were harvested by mitotic shake-off following exposure to colcemid (30 min at 0.04 μg/ml). Harvesting was performed 1, 2, 3, and 4 hr after the 3 hr ara A treatment. An interval of 30 min was left between the final (i.e. 1hr) irradiation and the addition of colcemid to minimize the risk of scoring cells which

had been in mitosis at the time of irradiation. Cells were then exposed to hypotonic solution (7 min at 0.075 M KCl), fixed with ice-cold methanol : glacial acetic acid (3:1) and left overnight. After washing 3 times in fixative, suspensions were dropped on to ice-cold pre-cleaned slides and gently flame dried. This slides were then stained in 3% Giemsa for 10 min.

The numbers of chromatid aberrations were scored according to the criteria outlined by Savage (1975) as cited in Scott et al (1983).

5.2.7. *X irradiation*

As already described in section 2.3.

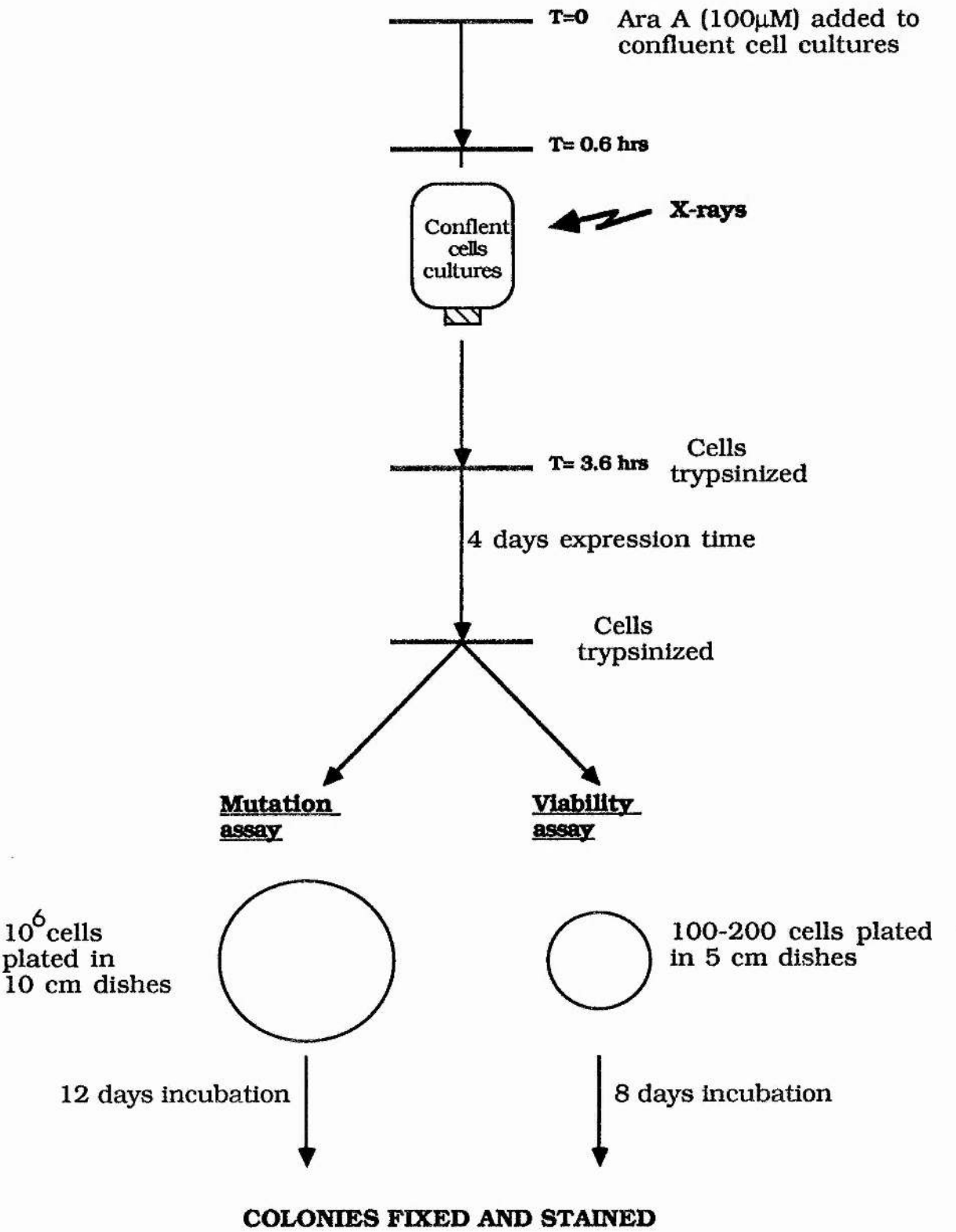


Figure 5.2. Summary of the modifications to the mutation assay

5.3 Results

5.3.1. *Inhibition of DNA synthesis using ara A in CHO KI cells*

The inhibitory action of the nucleoside analogue ara A on DNA synthesis was tested by measuring the extent of ^{3}HTdR (tritiated thymidine) incorporation into the DNA of CHO KI cells during a 10 minute pulse. The amount of ^{3}H-activity incorporated into the cells measured in the presence of various ara A concentrations is presented in figure 5.3.

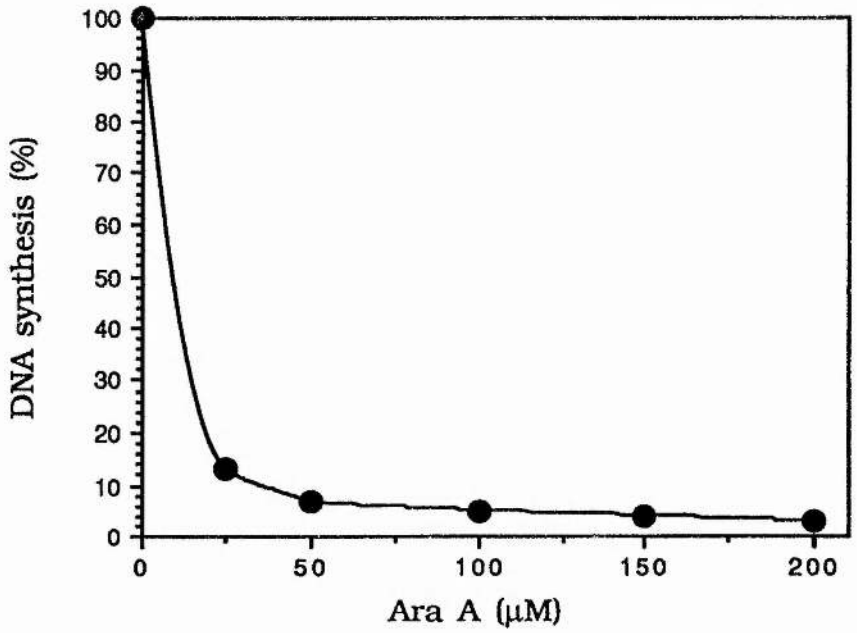


Figure 5.3. DNA synthesis assayed using ^{3}HTdR incorporation measured at increasing concentrations of ara A in exponentially growing CHO KI cells.

There was a strong decrease in the incorporation of ^{3}HTdR between the ara A concentration range of 0-25 μM. However beyond 50 μM, the incorporation of activity levelled off with no significant difference observed even at the highest concentration used (200 μM). Hence for all the mutation experiments using ara A, an intermediate concentration of 100 μM was chosen to achieve inhibition of DNA synthesis while reducing any possible toxic effects of the drug which might have arisen with the higher concentrations. Since the results

were obtained with exponentially growing cells, this implies that semi-conservative DNA synthesis can virtually be inhibited by the ara A concentration range of 50-200 μM. The incorporation of ^{3}HTdR in control samples (without ara A) was designated as 100% DNA synthesis and other values were represented as a percentage of this control value.

5.3.2. *Survival assay/CHO KI cells*

The results of the survival assay of CHO KI cells exposed to X-rays alone or in the presence of ara A (100 μM) are presented in figure 5.4. The assay was performed after the 3 hr post-irradiation incubation for both the samples exposed to X-rays alone or in the presence of ara A.

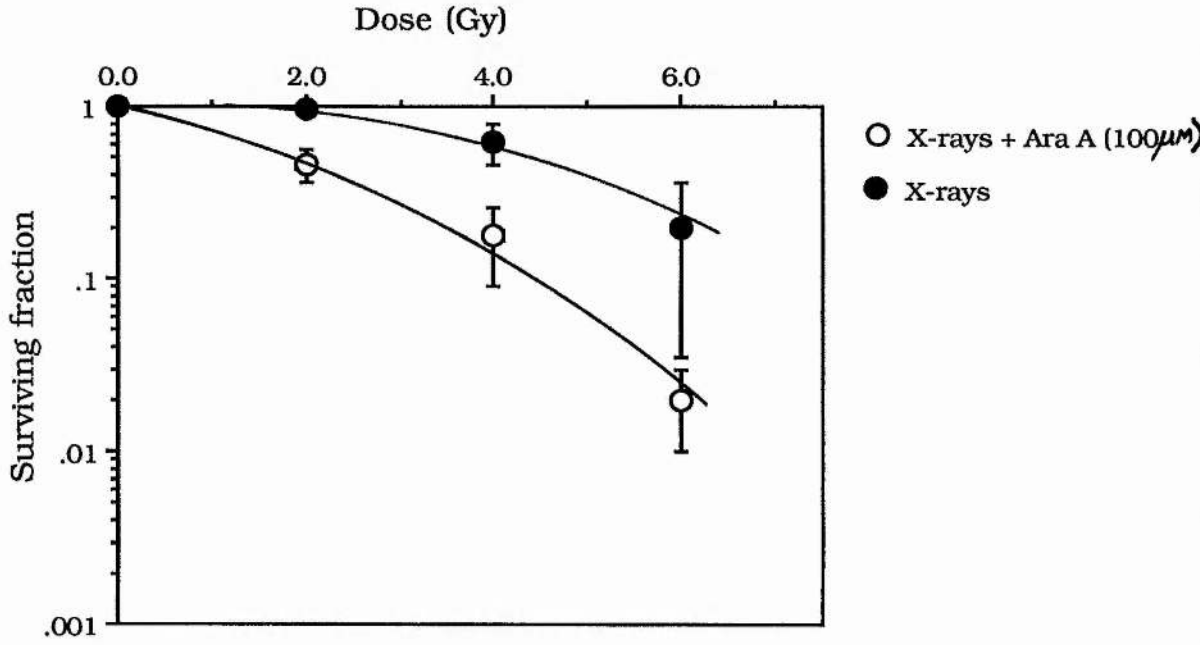


Figure 5.4. Survival curves of CHO KI cells exposed to X-rays alone or in combination with ara A. Vertical bars represent standard errors of mean values from three independent experiments.

Cell treated with ara A for 3 hr following irradiation showed a decreased survival relative to the untreated controls (X-rays alone). The survival curve of exponentially growing *xrs* 5 cells exposed to X-

rays only is plotted for comparsion in figure 5.5 together with the ara A and control data.

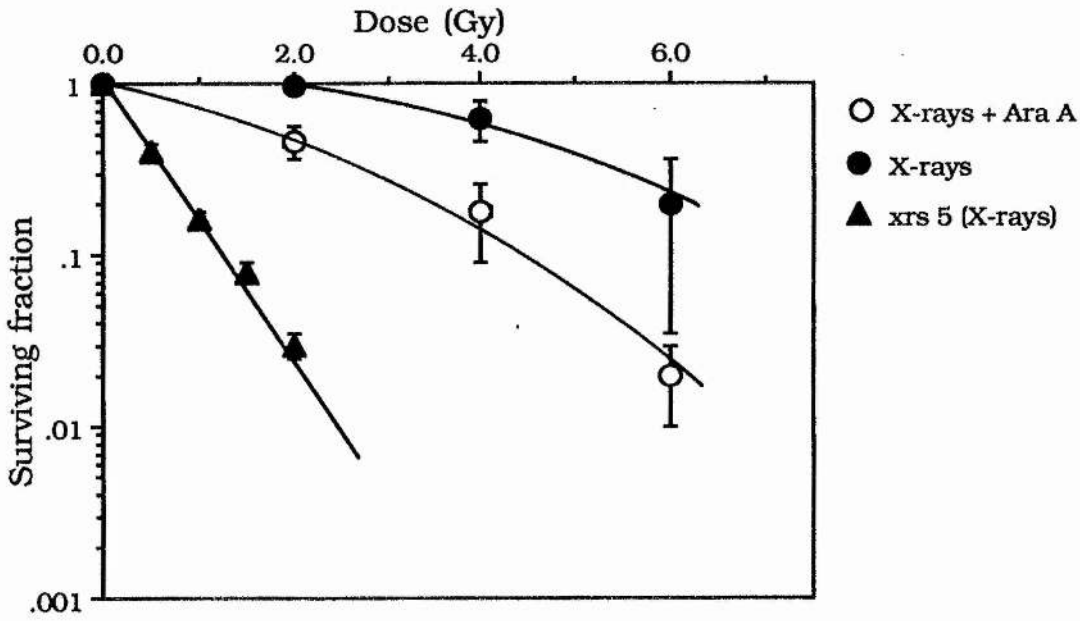


Figure 5.5. Survival curves of plateau phase CHO KI cells exposed to X-rays alone or in combination with ara A. Also included in the plot is the mutation data of *xrs* 5 cells exposed to X-rays alone. Vertical bars represent the standard errors of mean values from three independent experiments

The *xrs* 5 cells exposed to X-rays alone showed an exponential decrease in survival with increasing dose. In comparison, the CHO KI survival curves with or without ara A display a shoulder. CHO KI cells treated with ara A showed a shift towards the *xrs* survival curve, which may indicate a reduction in the shoulder associated with increased damage expression, or an inhibition of repair.

5.3.3. *Spontaneously induced mutation frequencies*

The results of the spontaneously induced mutation frequencies in CHO KI cells assayed following pre-treatment in HAT/MEM, are presented in table 5.1.

Treatment	Mutations per 10^5 survivors	Standard errors of mean values
Spontaneous	5.6	3.6
ara A (100 μM)	5.7	2.8

Table 5.1. Mutation frequencies for spontaneous and ara A control samples. Each value represents the average of at least three independent experiments.

The ara A control samples showed a mutation frequency of 5.7 per 10^5 survivors which was assayed in confluent cell cultures exposed to the drug alone (100 μM). This is similar to the spontaneously induced mutation frequency (5.6 per 10^5 survivors), both of which were measured after 4 days expression period. This low background of mutation frequencies is important in eliminating any errors in the quantitative calculation of mutation frequencies which might otherwise be present in cell lines with a high background mutation frequencies.

5.3.4. *Induced mutation frequencies in CHO KI cells*

The induced mutation frequency curves in CHO KI cells exposed to X-rays alone or in combination with ara A (100 μM), are presented in figure 5.6.

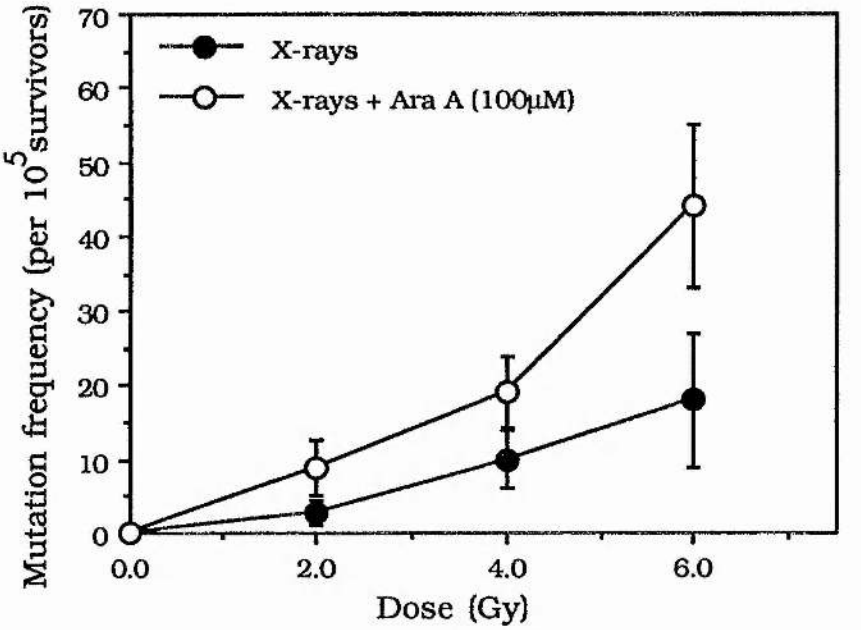


Figure 5.6. Mutation induction curves for CHO KI cells exposed to X-rays alone or in the presence of ara A. Vertical bars represent the standard errors of mean values from three independent experiments.

Plateau-phase CHO KI cells exposed to X-rays and post-treated with ara A show a 2-3 fold enhanced frequency of induced mutations compared to cultures exposed to X-rays alone (figure 5.6). The background mutation frequencies have been subtracted from the data points. In order to compare the effect of inhibition of dsb repair by ara A on mutation induction, the mutation data obtained following X-ray exposure of exponentially growing *xrs* 5 cells is replotted in figure 5.7.

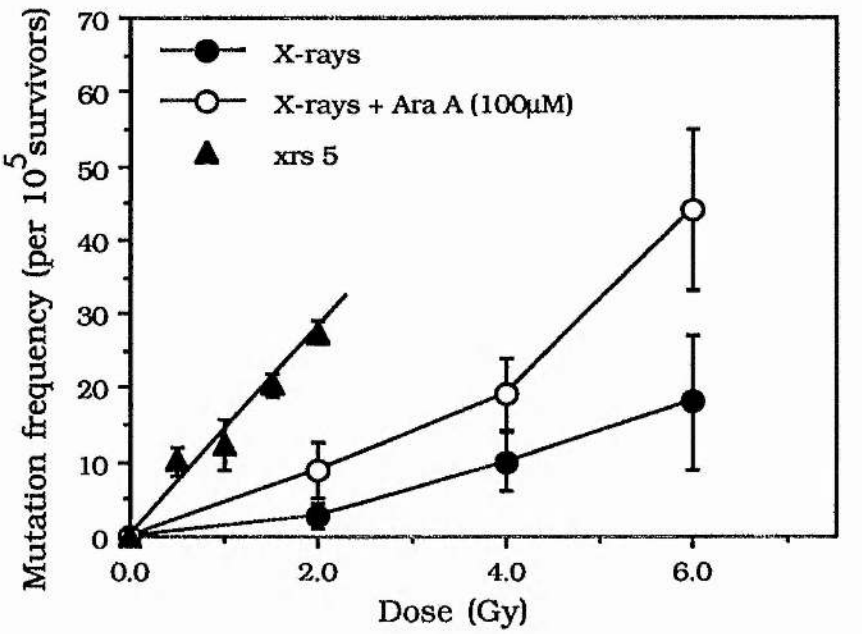


Figure 5.7. Mutation curves for CHO KI cells exposed to X-rays alone or in the presence of ara A. The *xrs* 5 mutation data in which exponentially growing cultures were exposed to X-rays is also included. Vertical bars represent the standard errors of mean values from three independent experiments.

The ara A mutation data does not show the hypermutability observed in the case of *xrs* 5 cells. However, this ara A curve represents an intermediate between conditions of normal repair (CHO KI cells) and dsb-repair inhibition (*xrs* 5 cells).

5.3.5 *Effect of ara A concentration on mutation induction*

Figure 5.8 shows the induced mutation frequency measured in plateau-phase CHO KI cells exposed to a single dose of X-rays (4 Gy) in combination with increasing concentrations of ara A (50-400 μM).

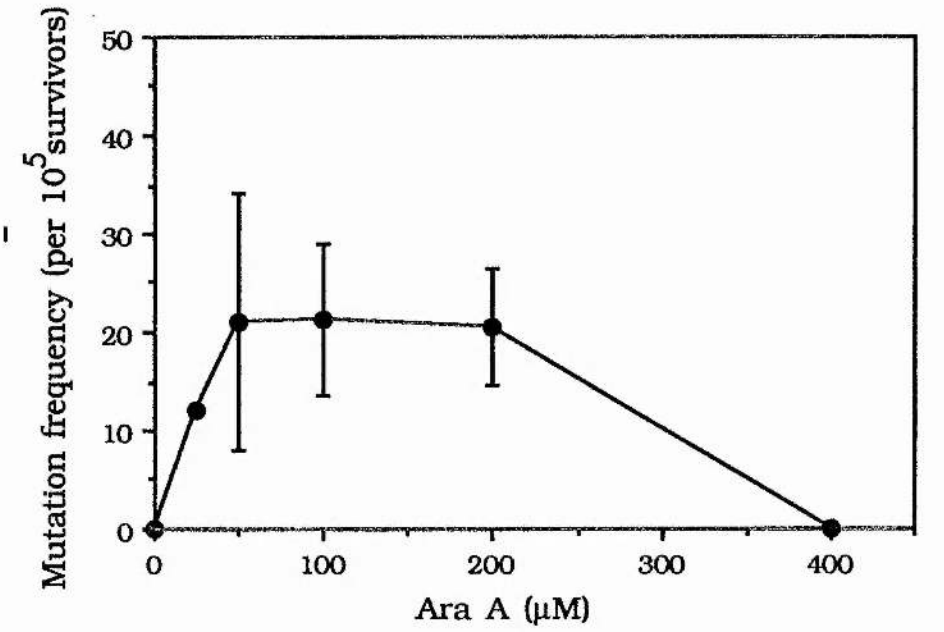


Figure 5.8. Effect of increasing concentration of ara A on the frequency of mutations in cells exposed to X-rays (4 Gy). Background mutation frequencies with the respective ara A concentration have been subtracted from the data points shown in the graph. Vertical bars represent the standard errors of mean values from three independent experiments.

The plot showed an initial near linear increase leading to a plateau, followed by a sharp decline in the induced mutation frequency at high concentrations. For all the data points shown in figure 5.8, the background mutation frequencies measured at the various concentrations alone (without X-rays) have been subtracted.

5.3.6 *Relationship between mutation frequency and cell survival*

A plot of induced mutation frequency versus log surviving fraction for CHO KI cells exposed to X-rays alone or in combination with ara A is presented in figure 5.9. The *xrs* 5 (exponential cells) mutation data from chapter 3 following treatment with X-rays has been replotted in the figure below.

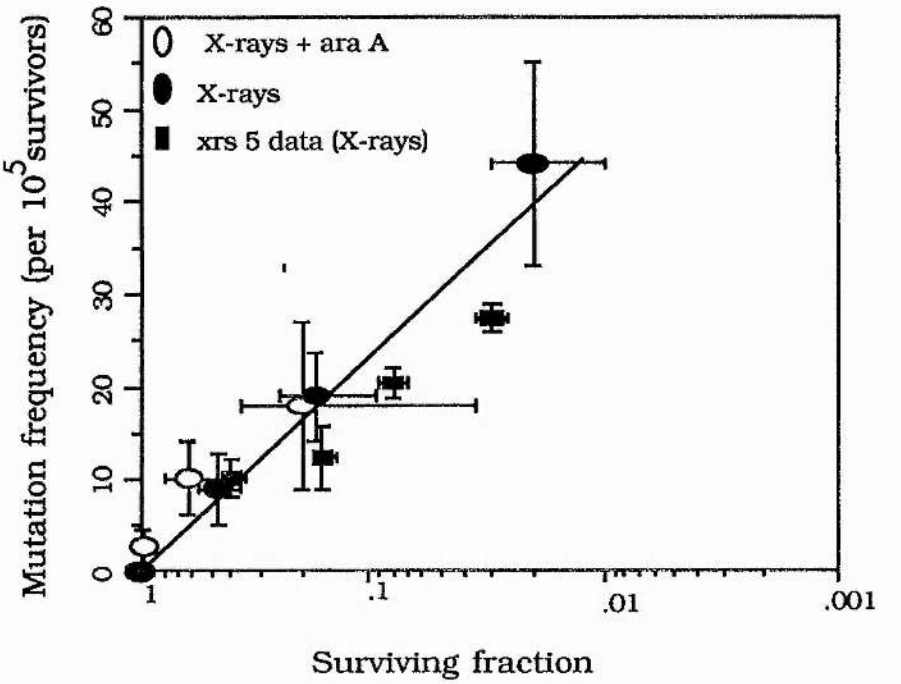


Figure 5.9. Plot of the induced mutation frequencies vs log surviving fraction after irradiation of CHO KI cells with X-rays alone or in combination with ara A. The *xrs* data from chapter 4 has also been replotted. Both the vertical and horizontal bars represent the standard errors of mean values from three independent experiments.

All sets of data show an increase in mutation frequency with a decrease in cell survival. A common line can be be fitted to all the three data points following the three different types of treatments conditions.

5.3.7. *Effect of ara A on the frequency of chromatid aberrations*
The results of the experiments in which cells were X-irradiated and incubated up to the time of fixation in the presence or absence of ara A, are shown in table 5.2 and figure 5.10.

Treatment	Time after irradiation before fixation (hr)	Aberrations per 100 metaphases		
		Gaps	Deletions	Total
Control	1	2	1	3
Ara A (control)	1	5	12	17
X-rays	1	96	292	398
X-rays	2	57	159	216
X-rays	3	33	87	120
X-rays	4	22	62	84
X-rays + ara A	1	125	293	418
X-rays + ara A	2	133	313	446
X-rays + ara A	3	118	283	401
X-rays + ara A	4	156	256	412

Table 5.2. Yield of chromatid breaks in CHO KI cells exposed to X-rays alone (0.75 Gy) or in the presence of ara A (100 μM). Data has been taken from Singh et al, 1990. Scoring was done by Predrag Slijepcevic.

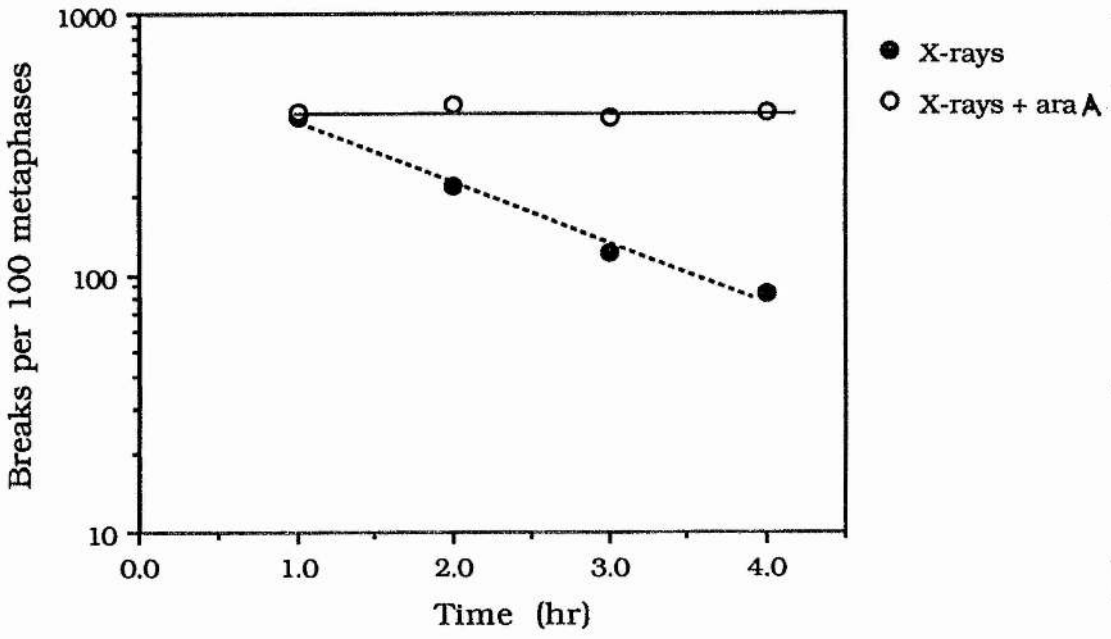


Figure 5.10. Frequency of chromatid aberrations as a function of time in cells exposed to X-rays alone or to X-rays and 100 μM ara A. Graph replotted from Singh et al, 1990.

For cells exposed to X-rays only, at 1 hr before fixation, the frequency of induced breaks per cell was approximately 40 breaks per 100 cells and the number of these decreased exponentially as the time of post-irradiation incubation was increased. In contrast, for cells exposed to X-rays at various times after treatment with 100 μM ara A and held in the presence of ara A from the time of irradiation until fixation, the frequency of breaks remained constant.

5.4 Discussion

The hypermutability observed in the radiosensitive *xrs* 5 cell line (chapter 4) implicated dsb as pre-mutational lesions which formed the basis of the experiments described in this chapter. The use of repair inhibitors for a specific lesion in an attempt to resolve the type of damage responsible for a defined biological endpoint has been widely exploited in a number of studies (Bryant, 1983., Bryant and Blöcher, 1982., Iliakis, 1980,1981., Mozdarani and Bryant, 1987). In the current experiments, the nucleotide analogue ara A, a drug known to be a potent inhibitor of DNA synthesis (Doering et al, 1966., Furth and Cohen, 1968., Müller et al, 1975), which also inhibits the repair of radiation-induced dsb (Bryant and Blöcher, 1982., Iliakis and Bryant, 1983) was used to study the effect of dsb-repair inhibition on mutation induction in CHO KI cells.

Biochemical studies have revealed that on entry into the cells, ara A is readily phosphorylated into the corresponding 5'-mono-, di or tri-phosphates (Brink and Le Page, 1965). This phosphorylated form of ara A is thought to bind to DNA polymerase sites competitively inhibiting the utilization of the normal substrate (dATP) thus resulting in a reduced DNA synthesis (Müller et al, 1975). A reduced incorporation of ^{3}HTdR was observed in exponentially growing CHO KI (figure 5.3) in the presence of increasing concentrations of ara A which is evidence for strong inhibition of semi-conservative DNA synthesis. A plateau in the DNA synthesis was observed beyond the concentration of 50 μM. The intermediate ara A concentration of 100 μM was used for all the mutation experiments described in this chapter to compromise between inhibition DNA synthesis (dsb repair) and cell killing which was found to occur at progressively higher concentrations. The use of this concentration for the mutation studies was consistent with other studies which have used a similar concentration range to inhibit repair of dsb in different rodent cell lines while measuring different biological end-points (Bryant and Blöcher, 1982., Iliakis, 1984).

Plateau-phase CHO KI cells irradiated in the presence ara A and held for 3 hr post-irradiation in ara A showed an increased radiation induced cell killing (Figure 5.4) with an apparently reduced shoulder region in the survival curve compared to cells exposed to X-rays alone.

The shoulder of the survival curves is often attributed to either an accumulation of sublethal damage or some saturable type of repair process (Alper, 1979). This increased radiosensitivity observed in cells treated in the presence of ara A has also been interpreted as the 'fixation' of potentially lethal damage (PLD) in irradiated cells (Iliakis, 1980). PLD, first described by Phillips and Tolmach (1966), was found to be repaired under post-irradiation conditions which allowed recovery from damage which otherwise is lethal in normal cells. Plateau-phase conditions were found to maximize the repair of PLD which was however inhibited in the presence of conditions which inhibit DNA synthesis eg. ara A, actinomycin-D etc. Based on the inhibition of dsb repair by ara A (Bryant and Blöcher, 1982), this decreased survival of ara A treated cells has been suggested as evidence for dsb as potentially lethal lesions. This is further supported by Bryant (1985), who in a study using restriction endonuclease treatment of permeabilized cells found an increased cell killing in cells treated with a restriction enzyme which induced blunt-ended dsb (Pvu II) which are thought to mimic radiation-induced dsb (Bryant, 1988, review). The enhanced radiosensitivity observed in exponentially growing *xrs* 5 cells (figure 5.10) is thought to be due to its inherent defective dsb repair ability (Kemp et al, 1984., Costa and Bryant, 1988) evidence implicating dsb in cell killing.

The repair of X-ray induced dsb has been shown to have similar kinetics to those of PLD repair (Blöcher and Pohlit, 1982), suggesting that the increased expression of PLD (figure 5.4) may be due to the inhibition of dsb repair. Hence stationary cultures were used for the mutation experiments in a further attempt to investigate the role of radiation-induced dsb in mutagenesis. Plateau-phase cultures of CHO KI cells exposed to X-rays in combination with ara A show an increased induction of mutations compared to cells exposed to X-rays alone (Figure 5.5). This increased mutation frequency observed in CHO KI cells in the presence of ara A is thought to reflect additional mutations which arise due to the absence (or reduced level) of dsb repair. This result is similar to the increased frequency of mutations observed (at the *hprt* locus) in EAT cells exposed to X-rays in the presence of ara A (Iliakis, 1984).

Misrepair broadly represents one of the main mechanisms by which most mutations arise e.g. loss of some base pairs during

rejoining of a break would result in a deletion classified under a frameshift mutation. The enhanced mutation frequency in ara A treated CHO KI cells would suggest misrepair of dsb as a critical step in the induction of mutations. This result may also suggest the existence of two mechanisms for dsb repair, one consisting of a pathway which is DNA synthesis dependent, allowing a correct rejoining of dsb, while the second involves a DNA synthesis independent repair process which may result in the misrepair of dsb, resulting in the increased frequency of mutation induction; hence it could be suggested that dsb may follow a particular repair pathway on the basis of differential requirements of DNA polmerization. In addition to this, the fate of a dsb may be dependent on its specific location in the DNA.

Based on the data obtained with ara A treated CHO in the present chapter, the increased radiosensitivity of ara A treated cells (figure 5.4) correlates with an increased frequency of mutation induction (figure 5.6). This correlation provides further support for the notion of a sub-grouping within the initial pool of induced dsb which would hence involve two types of dsb-repair mechanisms, one which results in non-repair of dsb giving rise to cell death while the other involving misrepair of dsb which may give rise to non-lethal mutations.

The *xrs* mutation data from chapter 3 has been redrawn in figure 5.7 to allow a comparison with ara A mutation data. The mutation curve of CHO KI cells treated with ara A show a shift towards the *xrs* 5 curve. The reason for the difference between ara A and *xrs* 5 mutation curves despite inhibition of dsb repair in both cases is not understood. Two possible reasons which could account for this would be either an incomplete inhibition of dsb repair with ara A or death of some presumptive *tk*- mutant cells due to the toxic effects of ara A. To test for this possibility, CHO KI cells were exposed to X-rays (4 Gy) in the presence of increasing concentrations of ara A (50-200 μM) and the corresponding mutation frequency measured (Figure 5.8). The initial linear increase in mutation frequency between the ara A concentration of 0-100 μM would reflect a gradual inhibition of dsb repair which are critical in the steps resulting in the final induction of mutations. This view is supported by Bryant and Blöcher, 1982 who, using the unwinding method, showed a reduced repair of dsb in the

presence of increasing concentrations of ara A. Based on this observation, further inhibition of dsb repair in the presence of increasing ara A concentrations (100-200 μM) does not give rise to any significant corresponding increase in mutation induction. The kinetics of the curve provides support for the use of the 100 μM ara A concentration. This concentration would also reduce any the toxic effects of ara A which may be evident at higher concentrations (e.g. 400 μM), resulting in cell death of any *tk*- mutant cells.

Based on the above results, the lower mutation frequency in ara A treated cells in comparison to the *xrs* 5 mutation data in exponentially growing cells (figure 5.7) may be explained as being due to a lower inhibition of dsb repair (compared to *xrs* 5 cells) in CHO KI cells by ara A, rather than any cell death at 100μM. This is not surprising since there is evidence which suggests that even in the *xrs* 5 cells, the repair of dsb is not totally defective (Kemp et al, 1984., Costa and Bryant, 1988) hence it may not be possible to inhibit dsb repair (to an level observed in *xrs* 5 cells) in CHO cells without increasing the toxic effects of ara A.

A plot of the induced mutation frequency vs log surviving fraction in CHO KI cells treated to X-rays alone or in combination with ara A is presented in figure 5.9. The data obtained with *xrs* 5 cells exposed to X-rays alone (from chapter 4) has also been replotted. All the sets of data can be fitted to an approximately linear relationship. Such a result has been found to be representative of other mutation systems in eukaryotic cells exposed to ionising radiation. This interpretation of such a plot can only be speculative at the present stage. However, it is interesting to note that all sets of data, where there is no inhibition of dsb repair and in conditions where dsb repair is inhibited fit a linear relationship. A similar linear plot was also found for exponentially growing cells (CHO KI and *xrs* 5) exposed to X-rays (figure 4.6). Such a linear plot has been thought to suggest a common mechanism for the induction of a mutation in irradiated cells (Thacker and Cox, 1977). Based on the above view, it could be speculated that this linear plot may also represent the fixation of a common lesion (dsb) to a mutation in all the three treatments, assuming that the mechanism of mutation induction in all the three conditions is similar. Hence, this may be used as further evidence for the role of dsb as an important radiation-induced pre-mutational lesion.

The rate of rejoining for both chromosome and chromatid breaks has been shown to correspond closely to the slow rate of dsb repair measured in EAT cells (Bryant and Blöcher, 1980., Blöcher and Pohlit, 1982). This would suggest that the rejoining of G_2 chromatid breaks reflect the underlying repair of a sub-class of dsb, although the observed frequencies of induced chromatid and chromosome breaks are much lower than the corresponding frequencies of dsb measured by biochemical means. CHO KI cells exposed to ara A between irradiation and mitosis show a constant number of breaks suggesting inhibition in rejoining (figure 5.10). Futhermore, this increased number of chromatid breaks in the presence of ara A provides evidence for inhibtion of dsb repair by ara A in the mutation assay, since the conditions during both treatments (G_2 and mutation) were kept identical. This inhibition of rejoining of breaks by ara A reflects the response of X-ray induced dsb in cells exposed to ara A where a strong inhibition of repair was observed (Bryant and Blöcher, 1982). This would provide further evidence for the suggestion that chromatid breaks parallel the repair of dsb. Based on these assumptions, the increased induction of mutations in ara A treated cells (CHO KI) and the high frequency of chromatid breaks in ara A treated cells (the experimental schedule for treatment with ara A was similar for both mutation and G_2 assay) suggests a similar mechanism by which inhibition of the sub-class of dsb repair leads to the induction of mutations and the formation of chromatid breaks, the latter may be lethal.

In the presence of ara A, irradiated cells would hold the induced dsb open, allowing misrepair of dsb. Molecular analysis of mutations induced following treatment with ionising radiation (70%) show large genomic deletions (Kavathas et al, 1980., Graf and Chasin, 1982., Thacker, 1986., Yandell et al, 1986., Liber et al, 1987). This parallels a corresponding increase in the number of chromosome aberrations (breaks/deletions) observed in cells deficient in dsb repair (Bryant et al, 1987). Both these observations support the hypothesis of the relationship between radiation-induced dsb, chromosome aberrations (e.g. non-lethal deletions) and mutagenesis.

CHAPTER 6

Use of Restriction enzymes to induce mutations

6.1 Introduction

The aims of this work were two fold: firstly to use restriction endonucleases (RE) causing dsb in DNA with various end structures, to induce mutations at the *tk* locus in CHO cells and in so doing to identify the type of lesion which may be critical in the steps leading to the induction of a mutation. Introduction of RE into cells was achieved via cell electroporation. The second aim was to investigate the type of damage at the gene level in the mutants induced following treatment with RE. To achieve this analysis at a molecular level, the first approach used was to transfect CHO *tk*- mutant cell lines with plasmid vectors containing the Herpes Simplex Virus *tk* gene (HSV-*tk*), an approach which has been successfully used to transfect rodent lines (*tk*-) to give rise to cells with a *tk*+ phenotype due to the exogenously provided HSV-*tk* gene (Wigler et al, 1977). The viral *tk* gene has been completely sequenced (McKnight and Gavis, 1980., Wagner et al, 1981) and thus provides an ideal target locus for the the induction and subsequent analysis of accompanying types of mutational changes. However, despite many experiments to transfect the *tk*- mutant cells with different plasmid vectors containing the HSV-*tk* gene by two different methods i.e. cell electroporation and calcium phosphate precipitation, the CHO *tk*- cells could not be successfully transfected to the *tk*+ phenotype. This transfection was attempted in two different laboratories with no positive result. The reasons for this are not understood but would suggest that following transfection, the HSV-*tk* gene is not successfully integrated and expressed in the genomic DNA in CHO cells. Due to this setback, it was decided to isolate *hprt*- mutants following treatment with a restriction enzyme in the Chinese hamster (V79-4) cell line. The choice of using the *hprt* locus was based on the extensive work already published involving mutation experiments at this locus (Caskey and Kruh, 1979, review). Furthermore cloning of complementary DNA (cDNA) sequence (Brennand et al, 1982., Koneiki et al, 1982) for the *hprt* gene has been achieved which has allowed an extensive study of molecular changes at this locus following different treatments (Thacker, 1986, 1990).

6.1.1 *Restriction endonucleases (RE)*

Restriction endonucleases are endo-deoxyribonucleases that hydrolyze phosphodiester bonds in double-stranded DNA. Isolated from a variety of bacteria, RE have the property to recognize and cleave specific DNA sequences. RE have been found to recognize tetra, penta- or hexa-nucleotide sequences in DNA (Roberts, 1987). They recognize and cut at restriction sites irrespective of the association of the DNA with proteins. Due to the specific type of damage induced by RE, Bryant (1984) suggested the use of RE to model radiation-induced dsb and showed that RE induce chromosome aberrations in a manner similar to that observed with ionising radiation. These similarities include the induction of chromosome-type aberrations during G_1 stage of the cell cycle and chromatid-type aberrations during both S and G_2 stages of the cell cycle (Obe and Winkel, 1985)

One of the major advantages of using RE is that unlike radiation which is known to generate a broad spectrum of damage (ssb, dsb, base damage, DNA-DNA and DNA-protein crosslinks), RE generate solely dsb, the influence of which one is enabled to study, without the interference of other lesions, on a defined biological endpoint. The end-structure of RE generated dsb has been shown to be crucial in the production of chromosome aberrations (Bryant, 1984). Blunt ended dsb of the type induced by Pvu II were shown to be much more effective in aberration induction than the cohesive variety e.g. those induced by Bam HI (Bryant, 1984). Based on these observations, various RE which produced different end-structures were used to examine the relative effectiveness of a specific dsb-type to induce mutations. Four RE were used during the course of the study, Pvu II, Eco RI, Hpa I, Xho I (Northumberland Biological Laboratories) all of which have the property to recognize a specific 6 base sequence. However, the recognition sequence and site at which these RE cuts within the genomic DNA to produce the dsb is unique to each RE (figure 6.1). Pvu II and Hpa I produce double-strand breaks with blunt ends whereas both Eco RI and Xho I produce cohesive-ended dsb with a 5 base overlap.

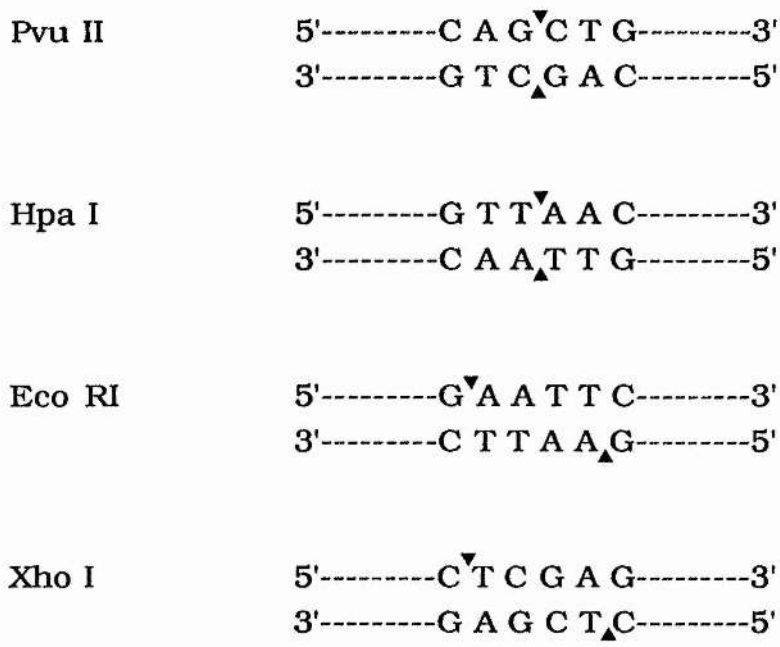


Figure 6.1. Recognition sequences and cutting sites for Pvu II, Hpa I, Eco RI and Xho I.

RE have now been extensively used to study chromosomal aberration formation (Bryant, 1984., Natarajan and Obe, 1984., Obe et al, 1985., Obe and Winkel, 1985., Gustavino et al, 1986., Winegar and Preston, 1988), oncogenic transformation (Bryant and Riches, 1990) and cell lethality (Bryant, 1985). Although the importance of dsb in radiation effects is better understood as a result of experiments using RE to mimic radiation damage (Bryant, 1984, 1985., Bryant et al, 1987), it is suggested that RE and radiation-induced lesions are not exactly similar and that both types of damage are thus handled differently by the cell during repair (Bryant, 1988, 1990). One of the reasons for this is that compared to radiation-induced dsb which are thought to be dsb with 'dirty' ends, RE generate 'clean' breaks with a 3'-hydroxyl and 5'-phosphoryl termini. This in turn will alter the way in which the two types of lesions will be repaired since the RE-induced 'clean' breaks in theory would require only a simple ligation process to rejoin while, the radiation induced 'dirty' ends would require exonuclease cleaning prior to the final step of ligation (Bryant,

1988). However, evidence for these two types of mechanisms is as yet lacking.

Despite the extensive use of RE in modelling radiation-induced damage, a major problem has been the introduction of RE into cells. A number of techniques have been used to permeabilise or 'porate' cells to RE, some of which include treatments with inactivated Sendai virus (Bryant, 1984), the cell 'pellet' method (Obe et al, 1985., Bryant and Christie, 1989) hypertonic and hypotonic shock (Winegar and Preston, 1988) and electroporation (Winegar et al, 1989). These widely different methods may explain some of the inconsistencies in results. This is highlighted in controversy over which types of dsb are more effective in generating chromosomal aberrations. Some workers have found blunt-ended to be more effective than the cohesive-ended dsb in inducing chromosomal aberrations (Bryant, 1984., Natarajan and Obe, 1984., Bryant et al, 1987) whereas others have found both types of end structured dsb to be equally effective (Gustavino et al,1986., Winegar and Preston, 1988).

6.1.2 *Electroporation*

Winegar et al (1989) showed that electroporation can be used as a rapid and efficient method for introducing RE into cells to induce chromosomal aberrations. The molecular mechanism by which RE enters the cell during cell electroporation is not well understood, however the basic principle involves the exposure of cells to a high-voltage electric discharge which has been shown to reversibly destabilize or 'porate' the membrane. This procedure which is known as cell electroporation has been found to be a rapid, simple and efficient method for introducing DNA into mammalian cells (Krutson and Yee, 1987., Neumann et al,1982). More recently, electroporation has been used to porate cells and introduce RE into cells to allow induction of micronuclei (Moses et al,1989). Cell electroporation was found to have an advantage over other techniques in that cell populations were found to be more uniformly permeabilised (Winegar et al, 1989).

The thinning of the membrane once a potential difference is applied across it (Knight, 1981., Potter, 1988) causes a reversible localized breakdown, forming pores (Knight, 1981). The size and life

time of these pores is sufficient to allow uptake of large molecules such as enzymes (Andreason and Evans, 1988) and even larger size DNA (Chu et al, 1987). In order to successfully electroporate cells without causing excessive cell death, it is important to optimize electroporation conditions for each cell type. The three parameters include the voltage of electric field, duration of pulse and composition of the electroporation buffer (Potter, 1988., Andreason and Evans, 1988).

For the electroporation in our laboratory, a Bethesda Research Laboratory (BRL) Cell-Porator™ electroporation system was used. The discharge of a capacitor through the cell suspension is limited by capacitance, resistance and conductivity of cell suspension. This interdependence of these three variables makes it difficult to exactly optimize the conditions. The cell suspension was placed in a disposable electroporation cuvette with a distance of 0.4 cm between the two electrodes. The field-strength (E) is calculated on the basis of the voltage settings and the distance between the two sides on the electroporation chamber hence E= V/d (0.4 cm). A schematic diagram of the electroporation setup is shown in figure 6.2.

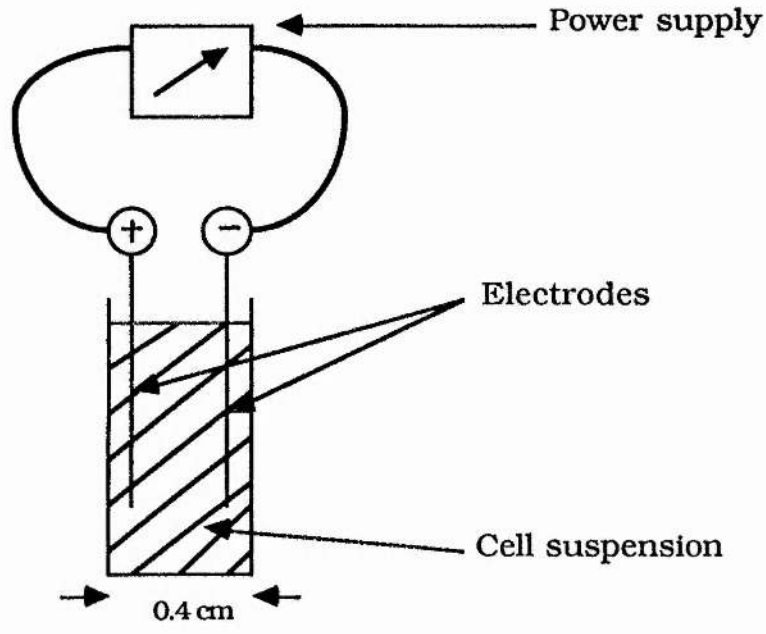


Figure 6.2. Schematic diagram of the Cell electroporation setup

In a preliminary study, CHO cells were permeabilised with inactivated Sendai virus and treated with Pvu II and Eco RI before assaying for the induction of *tk*- mutants. These results are discussed briefly to make a comparison with the electroporation data.

6.2 Materials and Methods

6.2.1 *Cell Cultures*

Two cell lines, CHO KI and the Chinese hamster (V79-4) were used for the experiments described in this chapter. Both cell lines were maintained in exponential growth in MEM/FCS as described in section 2.2.

6.2.2 *Purification of RE*

RE were generally stored at –20°C in storage buffer which contains 50% glycerol. This storage buffer was found to increase the background damage on its own (Costa, 1990a), probably due to the high glycerol content. Hence for all the experiments carried out, RE were purified free of storage buffer. Enzymes were purified using Amicon-10 ultrafilters to remove storage buffer (Bryant and Christie, 1989). The Amicon-10 filters were sterilized by rinsing twice with 70% alcohol followed by three rinses with double-distilled H_2O. The filters were initially saturated with bovine serum albumin (BSA) protein and kept on ice. Appropriate units of RE were placed on a filter and diluted with 1 ml HBSS. This was followed by centrifugation at 8000 rpm (Beckman Ultracentrifuge) for 1 hour at 2°C. This step was repeated twice before recovering the RE in 50 µl HBSS (achieved in an MSE Chillspin set at 2°C, 4000 rpm for 3 min). The purified enzymes were then usually diluted to 10 units/µl in calcium-free HBSS containing 6mmol/l $MgCl_2$ in 1% BSA and stored on ice.

6.2.3 *Cell Electroporation*

Exponentially growing cells were trypsinised from culture flasks and suspended in HBSS-BSA. Cells were centrifuged (1000 rpm, 5 min) and resuspended in HBSS-BSA twice. The final cell

concentration was adjusted to 10^6 cells per ml in HBSS-BSA. Purified RE were mixed with 1 ml of cell suspension (10^6 cells) in an eppendorf tube before pipetting into a disposable electroporation chamber. The Cell-porator™ was set to the following parameters: Field strength, 750 V/cm; Capacitance, 1600μf; Resistance, low; Temperature, ambient. The electroporation chamber was rinsed with HBSS-BSA between each enzyme treatment. Immediately after electroporation, samples were poured into 10 ml conical centrifuge tubes containing 5 ml of warm MEM/FCS medium. Samples in medium were then centrifuged (1000 rpm, 5 min) and the supernatent aspirated. Cells were resuspended in 10 ml of fresh medium, transferred into 75 cm^2 flasks (Sterlin) and incubated for 4 days at 37°C. The medium was changed after 24 hours. All electroporations were repeated twice for each dose point.

6.2.4 *Sendai virus permeabilization*

The permeabilization procedure was based on that used by Bryant (1984) and Bryant et al (1987) with a few modifications. Prior to treatment, 2 x 10^5 cells (CHO KI) were seeded in 5 cm dishes supplemented with 5 ml MEM/FCS. Dishes were incubated overnight at 37°C. This was followed by placing the dishes on ice, the medium was discarded and the dishes rinsed once with 1 ml of HBSS/BSA. To each plate, 300 μl of solution containing 400 Haemagglutinin units (HAU) of UV-light inactivated Sendai virus was added (virus inactivated by 3 KJ/m^2 ultraviolet light). This was followed by the addition of appropriate units of RE. All dishes were tilted to allow an even mixing and to allow RE/virus mixture to cover all the cells. The dishes were then left on ice for 10 minutes and returned to the incubator at 37°C for 30 minutes, the contents being mixed gently every 5 minutes to ensure that all cells were treated evenly. To avoid any trypsin induced damage, the dishes were rinsed twice with fresh MEM/FCS and incubated in 5 ml medium. Samples were held overnight at 37°C prior to trypsinising, and replating at 2 x 10^5 cell in 75 cm^2 flasks and for the four day expression time.

6.2.5 *Mutation assay*

Cells were plated out for the isolation of mutant cells (*tk*-) as described in section 2.4.

6.2.6 *Cell Transfection*

For the transfection of *tk*- cells (CHO *tk*- and murine Ltk-), two methods were used; Electroporation and calcium phosphate precipitation. Three plasmid vectors which contained the HSV-*tk* gene were used, namely: pFG5 (provided by Dr. Döhmer), pOT-TK (provided by Dr. Döhmer) and pRT (provided by Dr. Autonicn). The CHO *tk*- cells could not be transfected, while positive transfection with all the three plasmids was achieved in the Ltk- cells.

6.2.7 *Mutation assay to isolate hprt- mutants*

Exponentially growing Chinese hamster V79 cells were maintained and passaged in MEM/FCS as described in section 2.2. Before each mutation experiment, the cells were trypsinised and diluted to give 1000 cells per 25 cm^2 flask and allowed to reach a semi-confluent form. This procedure was adopted to reduce the number of spontaneously induced mutants in the initial culture. Each flask was trypsinised separately and resuspended in 5 ml MEM/FCS. The suspensions were diluted to give 10^6 cells per ml and transferred into Eppendorf tubes. To each cell suspension, 40 units of purified Pvu II was added and the suspension mixed by a gentle inversion. Suspensions were transfered into electroporation cuvettes and electroporated under similar conditions as described in section 6.2.3. Electroporated cells were transfered to 10 ml V-tubes and centrifuged at 1000 rpm for 5 minutes, supernatant was aspirated and pellet resuspended in 10 ml MEM/FCS. The cells were transferred to 75 cm^2 flasks (Sterlin) and incubated for a 5 day expression period (Dr. J. Thacker, personal communication). Medium was changed after the overnight incubation and the cells maintained in exponential growth during the expression period.

To isolate *hprt*- mutants, cells were plated at approximately 10^5 cells per 10 cm dish with 10 ml MEM containing 6-Thioguanine at a concentration of 1 μg/ml. The dishes were incubated for 12 days in a

humidified incubator at 37°C before isolating *hprt*- mutant colonies. For the viability assay, 100-200 cells were seeded in 5 cm dishes with normal medium (MEM/FCS) and incubated for 8 days at 37°C.

All the *hprt*- mutant colonies were isolated and frozen down as described (section 2.7 and 2.8) before extraction of DNA and subsequent analysis of mutant cells.

6.3 Results

6.3.1 *Pvu II/Eco RI induced mutations in CHOKI cells*

The induced mutation frequencies (*tk*-) in CHO KI cells treated with varying concentrations of both Pvu II and Eco RI are presented in figure 6.3.

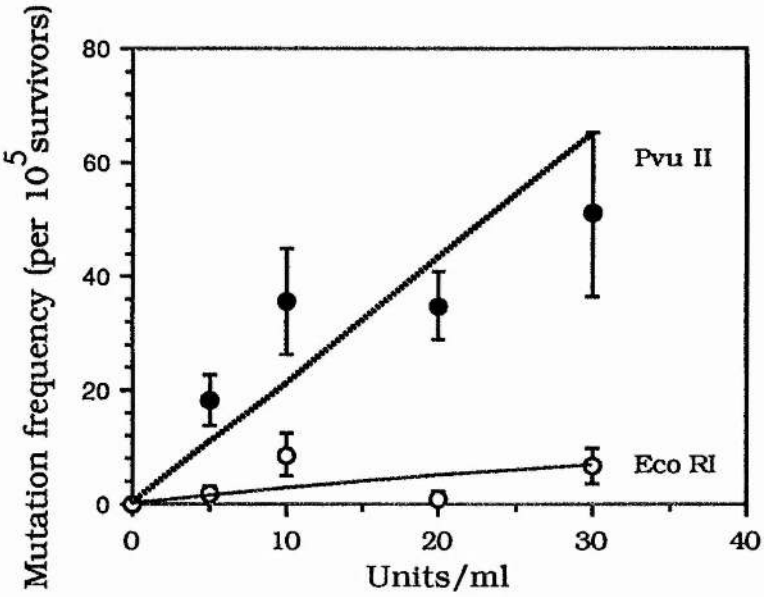


Figure 6.3. Induced mutation frequencies at the *tk* locus in CHO cells as a function of the concentration of Pvu II and Eco RI. All lines are fitted by eye and vertical bars represent standard errors of mean values from three independent experiments.

For both treatments, the frequency of induced mutations was enzyme-dose dependent. However, Pvu II was found to be more effective (by a average factor of ~9) than Eco RI in the induction of mutations at the *tk* locus. A straight line has been drawn by eye through the data points. The background mutation frequencies (table 6.1) have been subtracted from the value shown in figure 6.3.

6.3.2 Background mutation frequencies

The mutation frequency for both untreated and electroporated cells (without RE) are presented in table 6.1.

Sample	Mutation frequency (per 10^5 survivors)
Untreated cells	3.6
Electroporated cells	19.3

Table 6.1. Frequency of TFT-resistant mutations in untreated and electroporated samples. The values shown represent the average of three independent experiments.

The electroporated cells showed a 5-fold higher induced mutation compared to the spontaneously induced mutation frequency. However, despite this high background, the induction of mutations with RE were higher than this value which strongly suggests that the induction of mutations was due to the treatment with RE rather than the damage induced by the physical nature of the electrical discharge.

6.3.3 *Comparison of RE and X-ray induced mutations at the tk locus*

The X-ray induced mutation curve measuring mutations at the *tk* locus following exposure of exponentially growing CHO KI to increasing

doses of X-rays (2-6 Gy) has been replotted from chapter 4 to allow a comparison with the RE-induced mutation frequencies (figure 6.4)

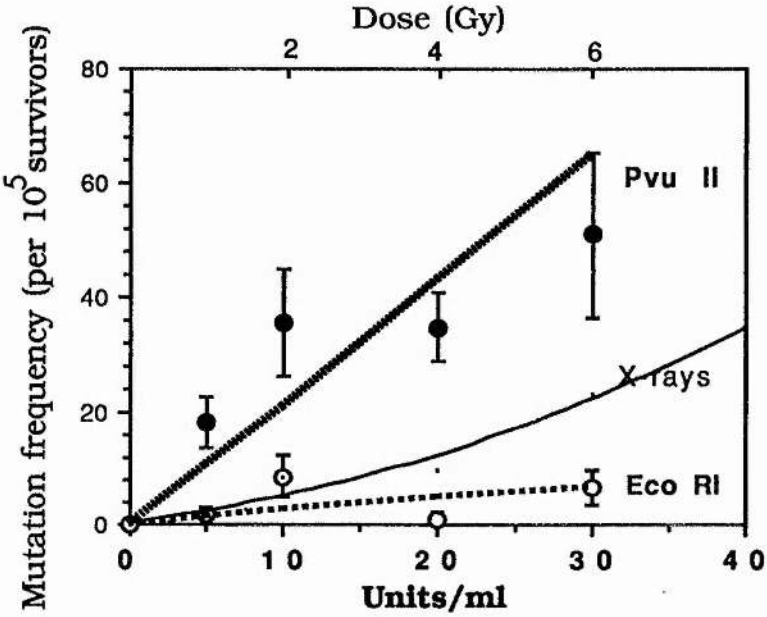


Figure 6.4. Induced mutation frequencies at the *tk* locus in CHO cells as a function of the concentration of Pvu II and Eco RI. A curve showing X-ray induced mutations is also shown (redrawn from Mussa et al, 1990). All lines are fitted by eye and vertical bars represent standard errors of mean values from three experiments.

The top horizontal axis represents the dose in Grays. The X-ray induced mutations show a curvilinear response in the induction of mutations which lies intermediate between the Pvu II and Eco RI mutation curves.

6.3.4 Effect of expression time on mutation induction

The induced mutation frequencies (at the *tk* locus) following treatment with Pvu II and Eco RI after an expression time of 7 days are presented in figure 6.5.

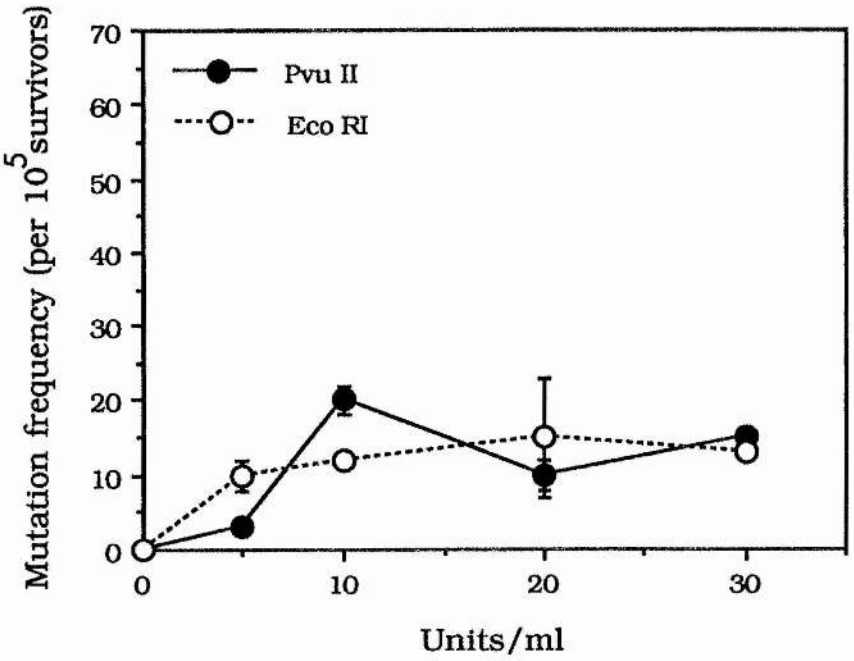


Figure 6.5. Induced mutation frequencies in CHO cells following treatment with Pvu II and Eco RI measured after a 7 days expression time. Vertical bars represent the standard errors of mean values from three independent experiments.

The Pvu II induced mutation frequencies show a decrease while the Eco RI data shows a slight increase compared to the mutation frequencies measured at 4 days expression time (figure 6.3). These lowered Pvu II induced mutations could be explained by cell death during this prolonged expression time while slow growing Eco RI-induced mutants are expressed and appear during this period. This observation may be indicative of major changes associated with Pvu II treatment which may account for the increased lethality during this period with a subsequently reduced mutation frequency. Based on this reduced mutation frequency, in all the RE-experiments, 4 days was used as the optimum expression time.

6.3.5. Hpa I/Xho I induced mutations in CHO KI cells

The induced *tk*- mutation frequency in CHO KI cells treated with various concentrations of purified Hpa I and Xho I are presented in figure 6.6.

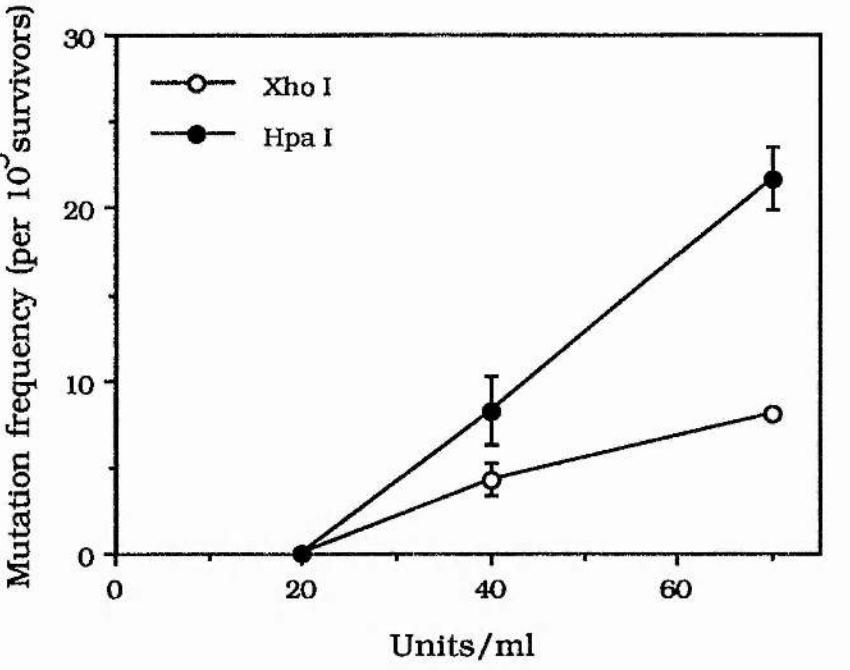


Figure 6.6. Induced mutation frequencies at the *tk* locus in CHO cells as a function of the concentration of Xho I and Hpa I. Vertical bars represent the standard errors of mean values from three experiments.

The enzyme Hpa I produces a blunt-ended dsb while Xho I produces cohesive-ended dsb. Both these RE have single restriction sites within the genomic *tk* gene (figure 6.14). The mutation frequency showed a dose-dependant increase following treatment with either RE, however Hpa I was more effective in inducing mutations on a per unit basis than Xho I. The background mutation frequency has been subtracted from the data points shown.

6.3.6. Comparison of mutation induction at tk and hprt loci

The mutation data in CHO KI and Chinese hamster V79 cells exposed to X-rays are presented in figure 6.7.

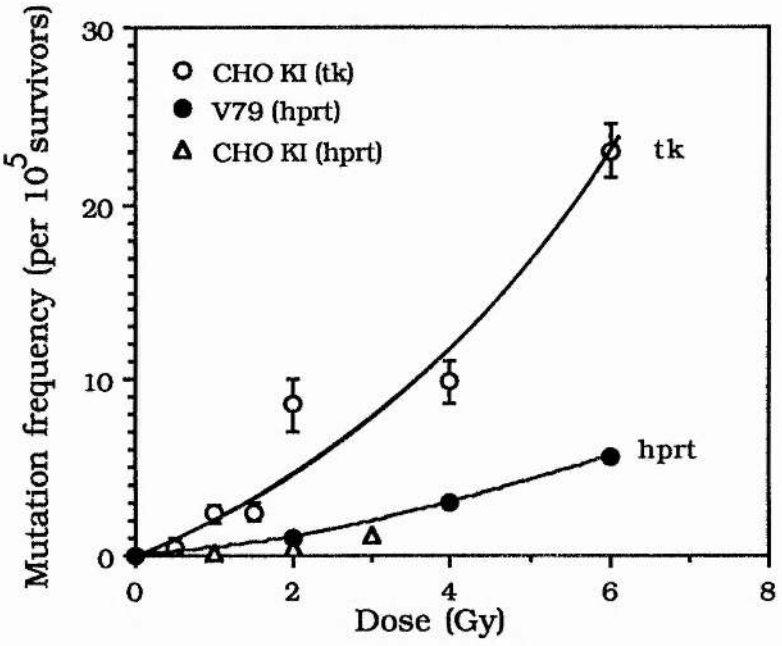


Figure 6.7. X-ray induced mutations at the *tk* and *hprt* loci [(V79/*hprt* data from Van Zeeland and Simons (1976) and CHO/*hprt* data from Darroudi and Natarajan, (1989)]

The heterozygous *tk* locus in CHO KI cells is clearly mutated at a higher frequency (factor of ~3) compared with mutations induced at the hemizygous *hprt* locus measured in both CHO KI and Chinese hamster V79 cells. A similar comparison was made between data for mutations at the *tk* and *hprt* loci following treatment with RE (figure 6.8).

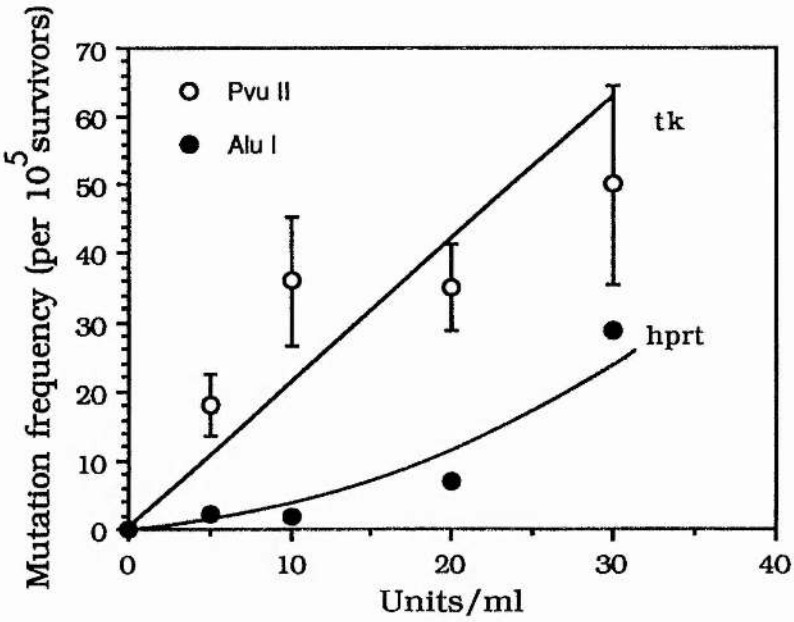


Figure 6.8. Induced mutation frequencies at the *tk* locus in CHO cells exposed to Pvu II and at the *hprt* locus in V79 cells exposed to Alu I (*hprt* data form Obe et al, 1986). Vertical bars represent the standard errors of mean values from three experiments.

Like X-rays, Pvu II induced mutations at the *tk* locus showed a similar higher mutation frequency (also a factor of ~3) in comparison with the *hprt*- induced mutation frequency in V79 cells exposed to Alu I, a RE which is similar to Pvu II, producing blunt-ended dsb.

6.3.7 ^{3}HTdR uptake in RE induced tk- mutant CHO KI cells

A mutant line induced and isolated following treatment with Pvu II (20 units) designated as as CHOtk-(Pvu II) was used to represent a prototype RE-induced *tk*- mutant cell line. The uptake of ^{3}HTdR was used as evidence of the loss of the thymidine kinase which is responsible for the uptake of the radioactivity via the salvage pathway. Both CHO KI and CHOtk- (Pvu II) cells are incubated in the presence of ^{3}HTdR, the uptake of which during DNA synthesis is measured at various sampling times (figure 6.9)

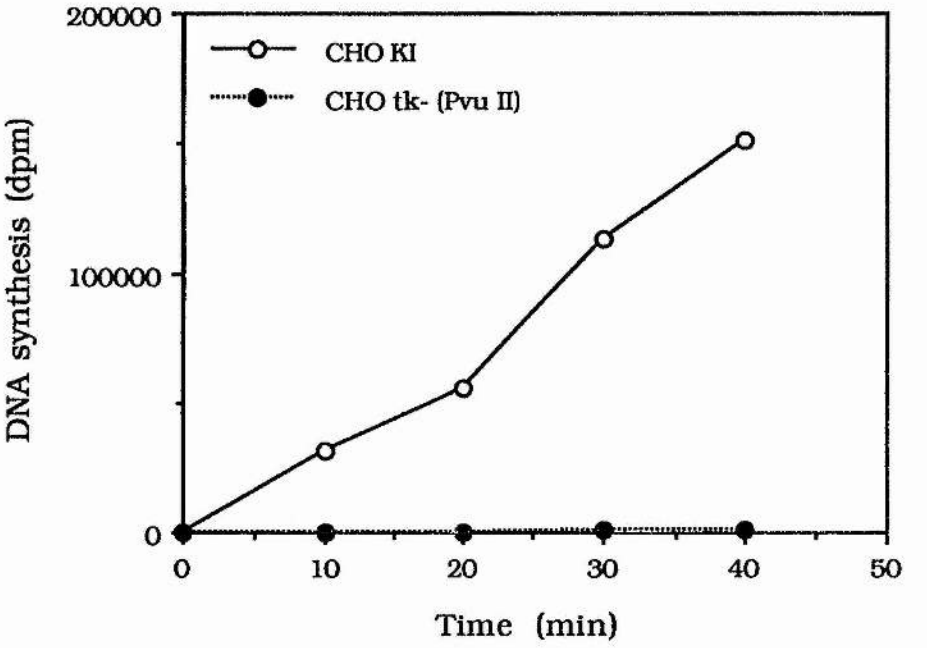


Figure 6.9. DNA synthesis measured as incorporation of ^{3}HTdR as a function of incubation time in both normal CHO KI and CHOtk- (Pvu II induced) mutant cell lines.

The normal CHO KI cell line shows a linear increase in the uptake of the ^{3}HTdR in comparison with the RE-induced mutant cell line which shows a complete lack of incorporated radioactivity. This observation provides evidence for a genetic change at the *tk* locus following treatment with RE resulting in the loss of active thymidine kinase. This result is similar to the radioactivity-uptake studies performed with a radiation-induced mutant, TK4 (section 3.3.4).

6.3.8 Use of Sendai virus to permeabilize CHO cells to introduce RE

The results of a preliminary study which involved the treatment of CHO KI cells with Pvu II and Eco RI introduced via Sendai virus permeabilization (Bryant, 1984., Bryant et al, 1987) are presented in figure 6.10. The mutation were measured at the *tk* locus.

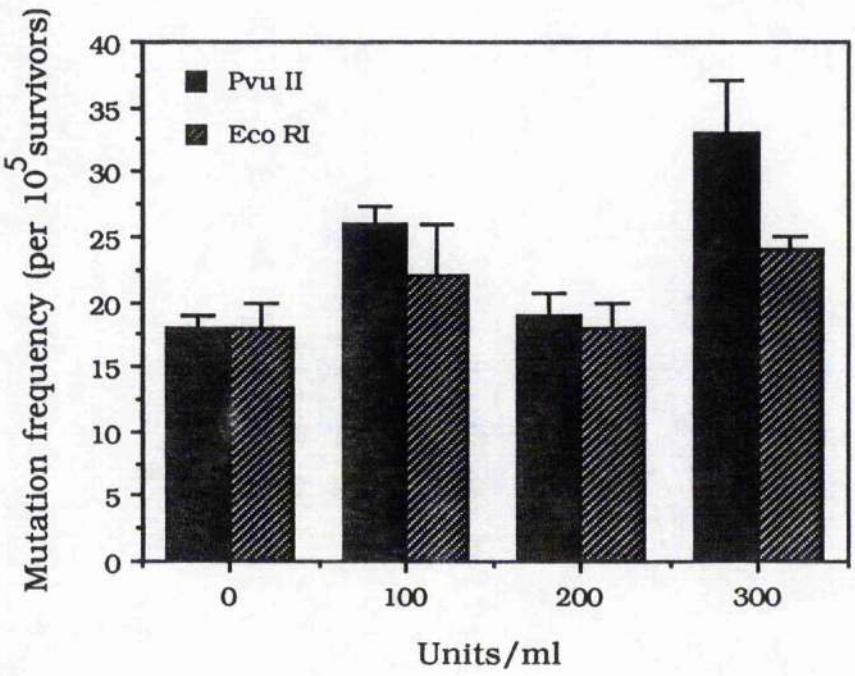


Figure 6.10. Induced mutation frequencies at the *tk* locus in CHO KI cells following treatment with equal units of Pvu II and Eco RI. Vertical bars represent the standard errors of mean values from three independent experiments.

Pvu II induced a higher mutation frequency as compared to Eco RI treated cells, a result which is similar to electroporation data (figure 6.3). However, the background mutation frequencies were very high (due to treatment with virus alone). Additionally, unlike results obtained with electroporation, no clear dose-response effect was observed and the reproducibility of the experiments was very poor. Due to the evident drawbacks of this procedure, all the experiments were carried out using electroporation as method for cell permeabilization due to the high reproducibility and increased induction of mutations obtained.

6.3.9 Background mutation frequencies (hprt-) in Chinese hamster V79 cells

The mutation frequencies for both control (spontaneous) and electroporated (without RE) samples measured at the *hprt* locus are presented in table 6.2.

Treatment	No. of mutant colonies/plates	Mutation frequency (per 10^5 survivors)
Control	0/15	0
Electroporated	4/20	0.25

Table 6.2. Frequency of *hprt*- mutations in untreated (spontaneous) and electroporated samples

The number of independent cultures and assays used to determine the background mutation frequencies included 20 plates for electroporated samples and 15 plates for the control samples. Both samples have an extremely low frequency of background mutations (0 and 0.25 per 10^5 survivors) which is important in reducing the probability of isolating a spontaneously induced mutant instead of a RE-induced mutant colony.

6.3.10 Pvu II induced mutations at the hprt locus in Chinese hamster V79 cells

The induced mutation frequencies following treatment of V79 cells with 40 units of purified Pvu II are presented in figure 6.11.

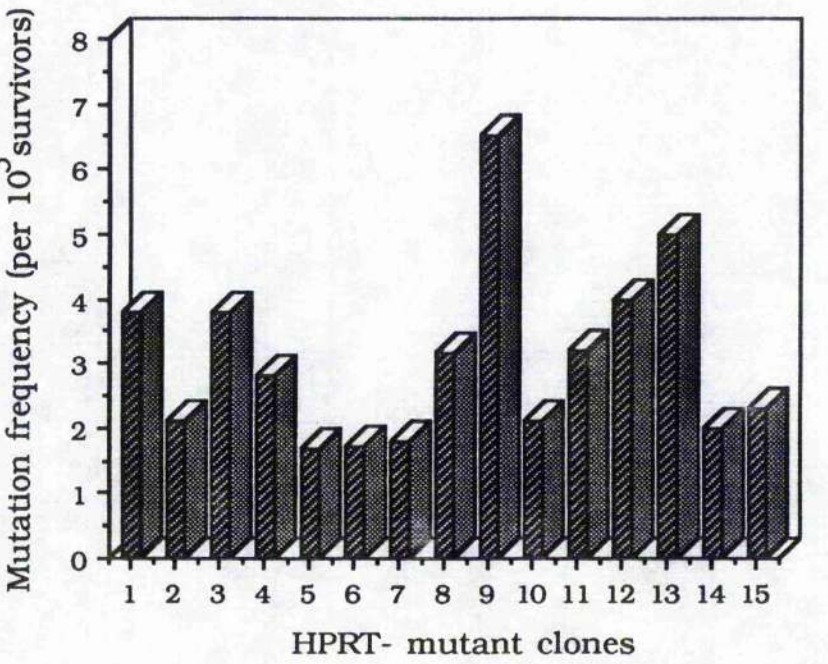


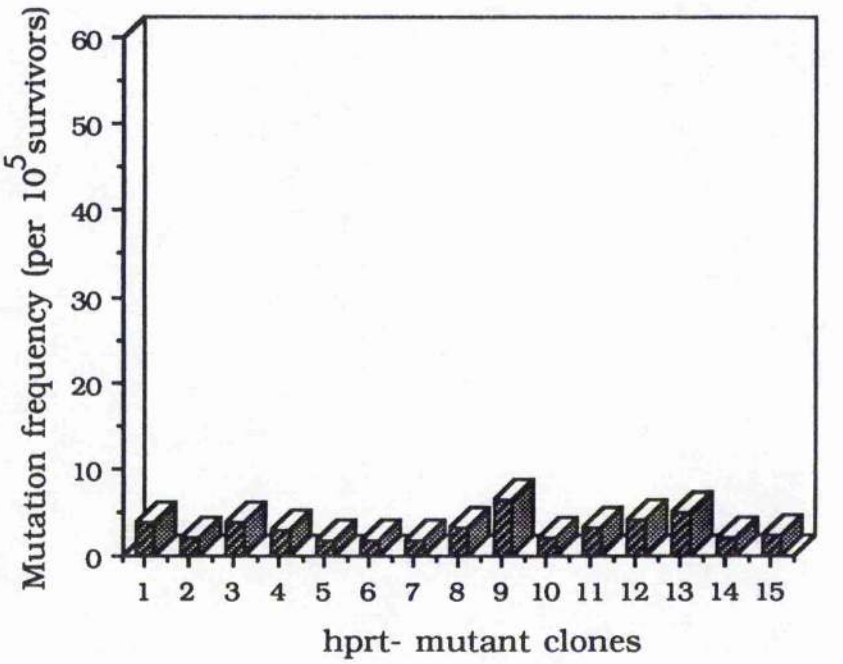
Figure 6.11. Induction and isolation of 15 mutants (*hprt*-) following treatment with 40 units of Pvu II. The background mutation frequencies have been subtracted from the values shown.

The mutation frequencies ranged from 1.5 to 6.5 mutations per 10^5 survivors in the mutants isolated. These values are 10-20 fold higher than the background mutations (0.25) which is strong evidence for the induction of mutants due to Pvu II treatment. This is important during the subsequent molecular analysis of the mutant clones (southern blotting and PCR).

6.3.11 RE-induced mutations at the tk (in CHO KI cells) and hprt locus (in V79 cells)

A comparison of the induced mutation frequencies measured at the *hprt* and *tk* cells following treatment with RE (40 units of Pvu II were used to isolate mutations at the *hprt* locus while 5-30 units of both Pvu II and Eco Ri were used to induce mutations at the *tk* locus) is presented in figure 6.12. A high frequency of mutations were recovered at this locus (Figure 6.12, lower panel) in comparison with

those at the *hprt* following RE treatment (figure 6.12, upper panel).



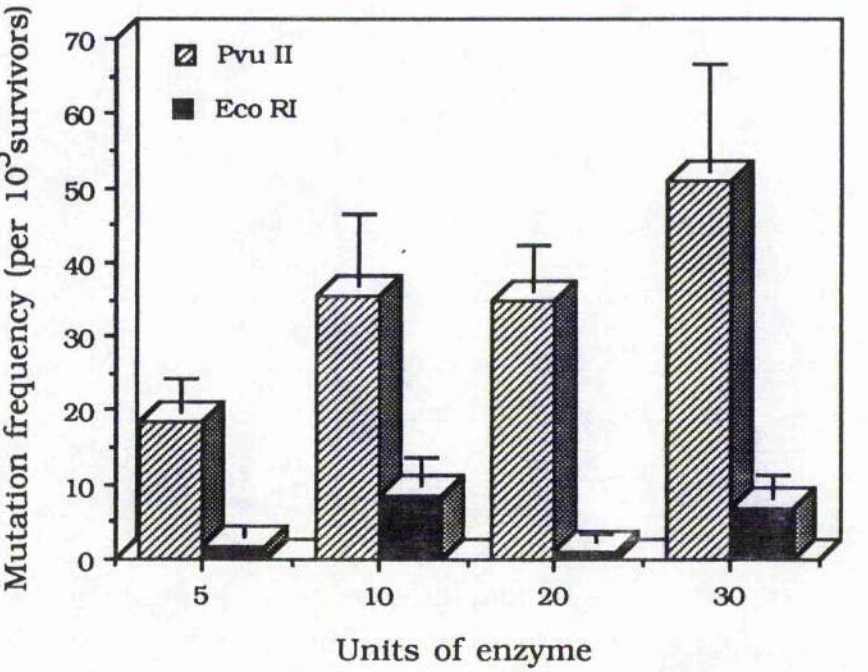


Figure 6.12. (a) *hprt* induced mutations in V79 cells following treatment with 40 units Pvu II (b) *tk*- mutations in CHO KI cells following treatment with 5-30 units of both Pvu II and Eco RI.

6.3.12 Approximate restriction map of the CHO KI genomic tk gene
The restriction sites of the RE used for the mutation assay within the genomic *tk* gene are shown in figures 6.13 and 6.14. Both the approximate sizes have been redrawn from Lewis (1986).

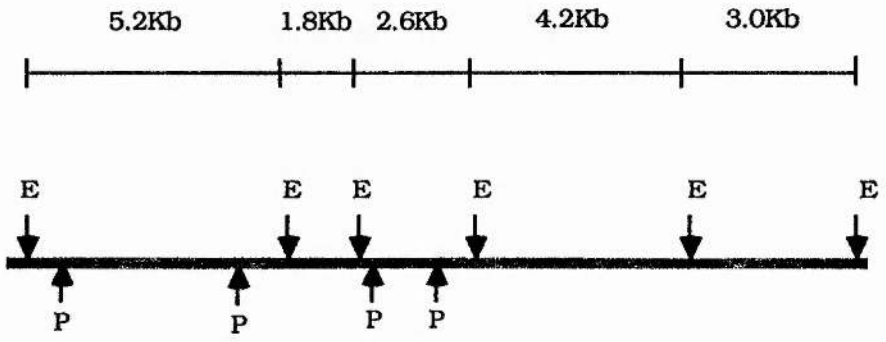


Figure 6.13. A Restriction map of the Chinese hamster *tk* gene showing Pvu II and Eco RI sites. The size of the Eco RI fragments is also shown.

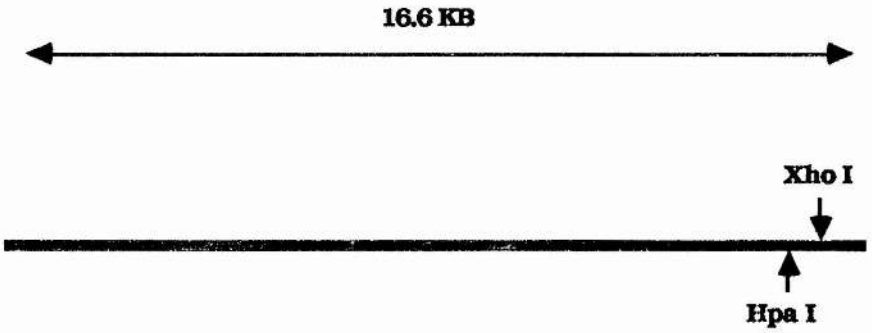


Figure 6.14. A Restriction map of the Chinese hamster *tk* gene showing the single restriction sites of both Hpa I and Xho I

6.3.13 Analysis of hprt- mutants

(a) Southern blot analysis:

To analyse the molecular changes associated with dsb, 15 **independently** isolated mutant cells (induced following treatment with Pvu II) were probed for the changes in the *hprt* gene. The induced mutants were selected from populations which gave approximately 20-30 % survival. The prefix 'M' indicates Pvu II induced mutants. Blots were probed with a full-length Chinese hamster cDNA, isolated from the plasmid pHPT12 (Konecki et al, 1982). The restriction fragments on southern blots of the mutants analysed are presented in figure 6.15-6.17. The methods used during the isolation and analysis of the mutant DNA were similar to those described by Thacker (1986).

(b) PCR:

The primers used for this study are those described in the following paper:

Exons 3, 5 and 9 (Rossiter et al, 1991)

The PCR conditions used are as follows (25µl or 100µl volume):

25ng/µl DNA
0.01 units/µl Taq DNA polymerase (BRL)
4 mM $MgCl_2$
10 mM Tris-Hcl, pH 8.3
50 mM KCL
0.1% NP40
100µM dNTPs (ultrapure, Phramacia)
400nM Primers

PCR programme:

94°C, 4.5 min
(60°C, 50 sec., 72°C, 2 min., 94°C, 30 sec) x 29 cycles
60°C, 50 sec
72°C, 7 min
refrigerate

Samples run on 2% agrose gel (figure 6.18-6.20).

The Southern blot and PCR analysis of the mutant cells was carried out in the laboratories of Dr. Thacker, MRC Radiobiology Unit, Harwell.

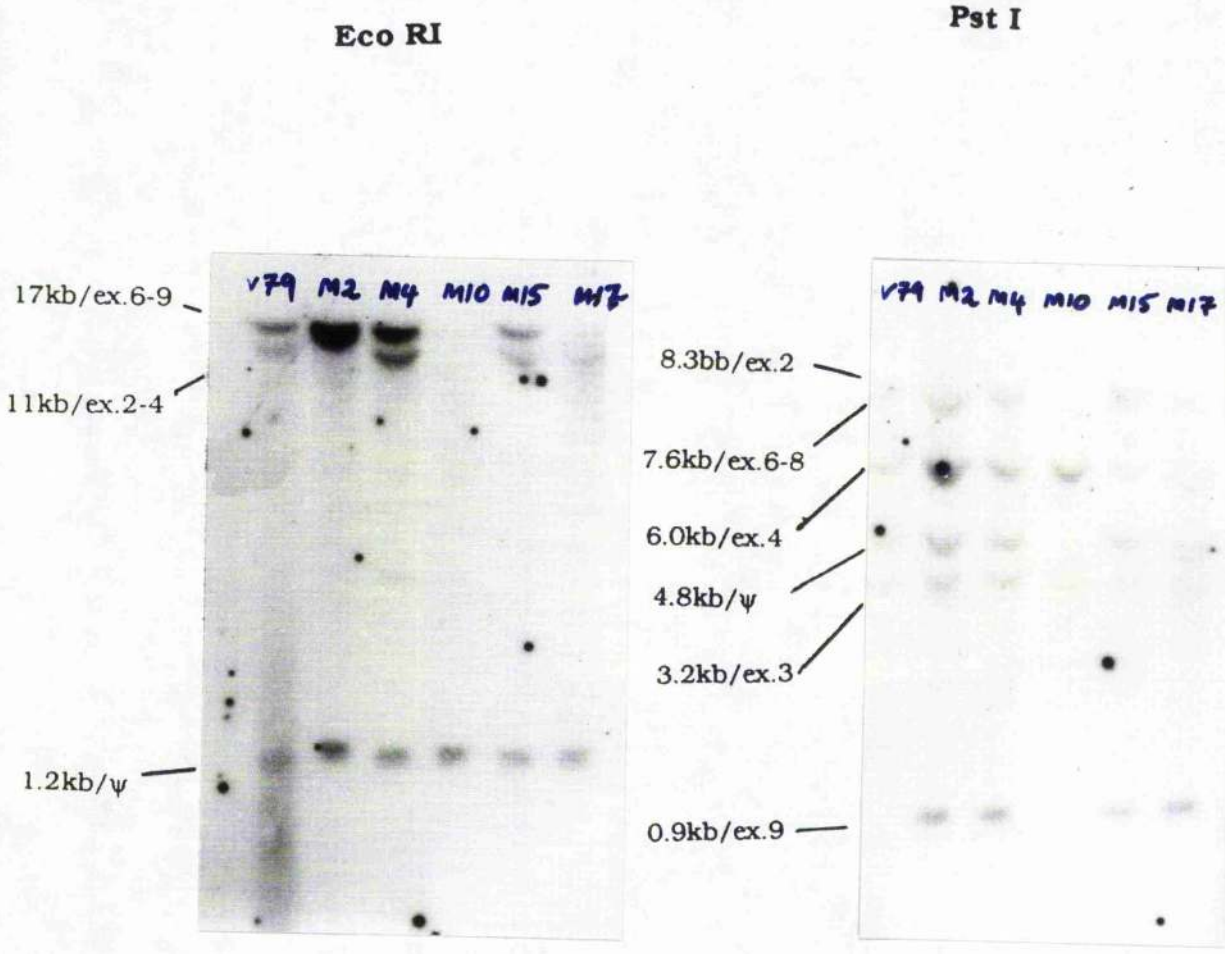


Figure 6.15. Southern blot showing parent (V79-4) and mutant *hprt* gene fragments for five Pvu II induced mutants (M2, M4, M10, M15, M17). To the left of each pattern is given the size of the fragments in kilobases (kb) and exons (ex.) contained in that fragment (Rossiter, 1987). Ψ represents the pseudogene fragment (Fuscoe et al, 1983., Thacker, 1986) (a) The pattern following digestion with Eco RI in the parent V79 cells show two bands in addition to the pseudogene fragment. The mutant M10 shows a complete loss of the two fragments while M2 shows loss of one band (b) Digest after treatment with Pst I, M10 shows the loss two/three bands.

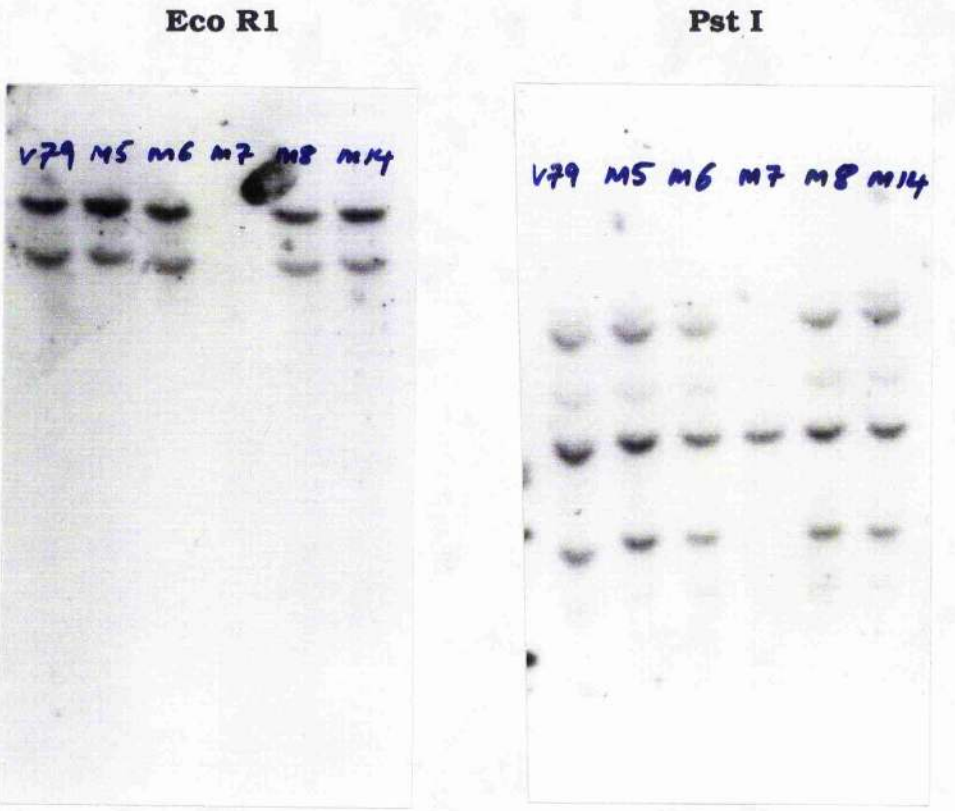


Figure 6.16. Southern blot showing parent (V79-4) and mutant *hprt* gene fragments for five Pvu II induced mutants (M5, M6, M7, M8, M14)). (a) The pattern following digests with Eco RI in the parent V79 cells show two bands in addition to the pseudogene fragment. The mutant M7 shows a complete loss of the two fragments (b) Digest after treatment with Pst I, M7 again shows the loss two/three bands.

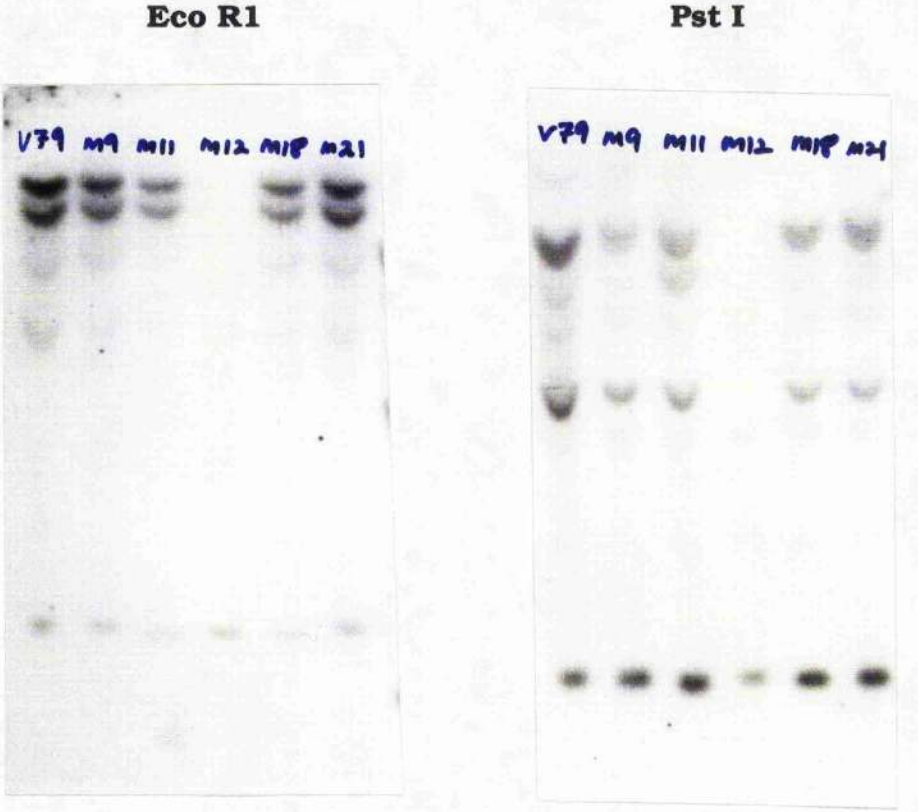
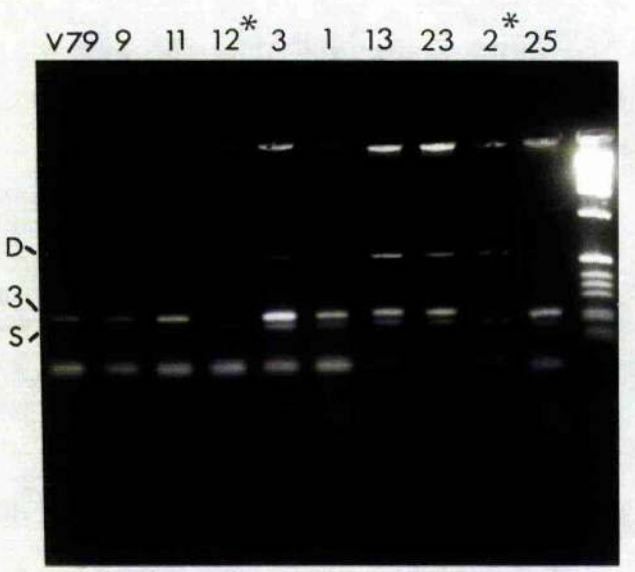
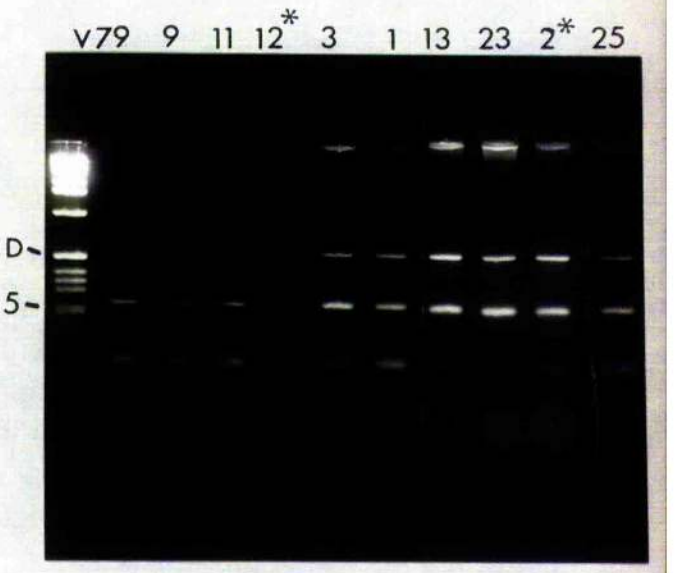


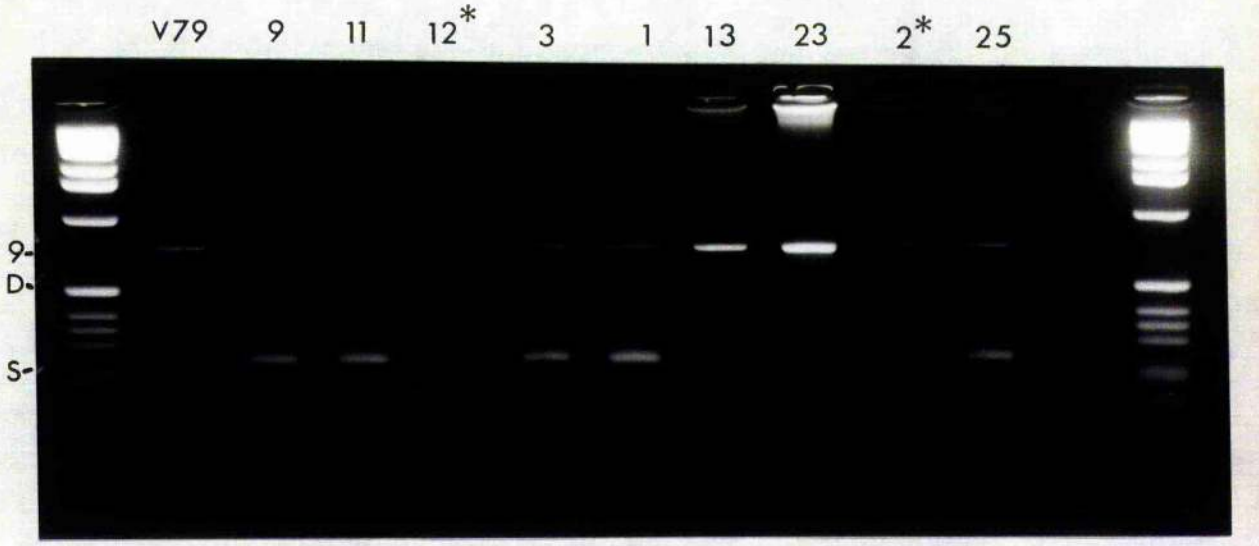
Figure 6.17. Southern blot showing parent (V79-4) and mutant *hprt* gene fragments for five Pvu II induced mutants (M9, M11, M12, M18, M21). (a) The pattern following digests with Eco RI in the parent V79 cells show two bands in addition to the pseudogene fragment. The mutant M12 shows a complete loss of the two fragments (b) Digest after treatment with Pst I, M12 shows the loss two/three bands.



3 + D



5 + D



9 + D

Figure 6.18. For each gel, an internal control for the Duchene Muscular Dystrophy (DMD) was amplified as a control, S represents supurious products, 3=exon 3 (220 bp), 5=exon 5 (247 bp) and 9=exon 9 (743 bp), **3 + D**= amplification of exon 3 and DMD gene, **5 + D**= amplification of exon 5 and DMD gene, **9 + D**=amplification of exon 9 + DMD gene.

(a) M2 has lost exon 3 while exon 5 and 9 are present

(b) M12 shows a loss of all three exons (3, 5 and 9).

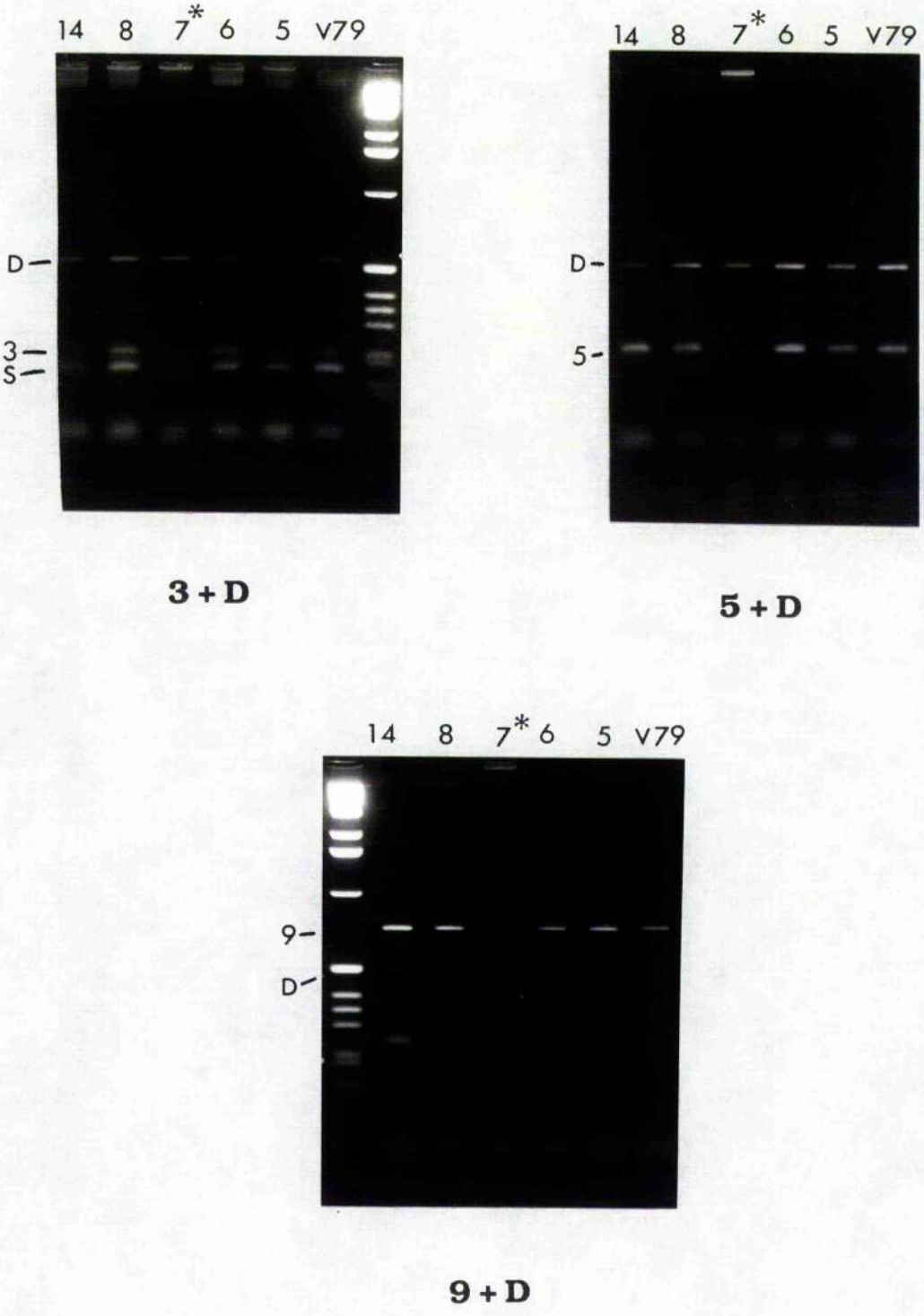


Figure 6.19. (a) M7 has lost all the three exons (3,5 and 9).

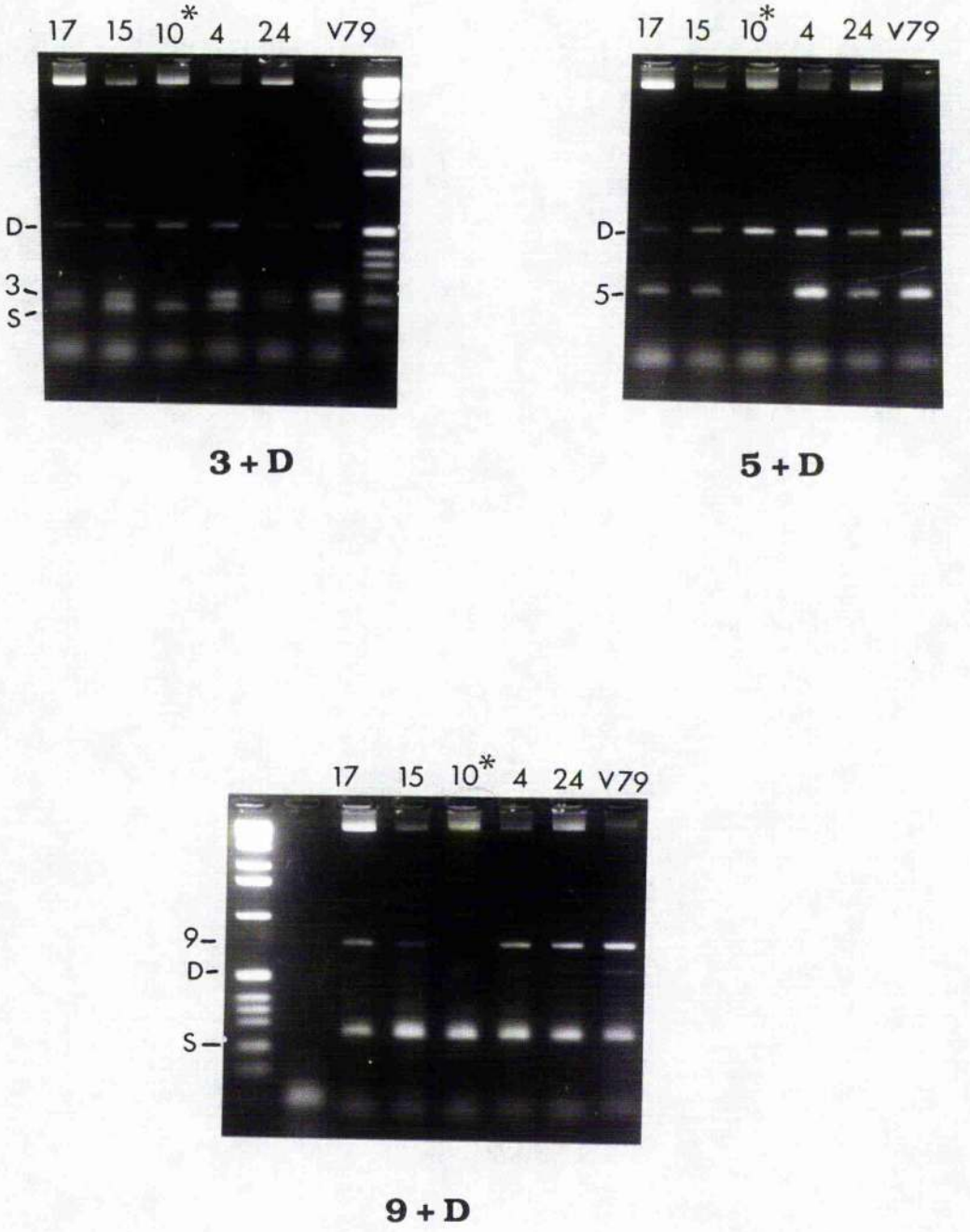


Figure 6.20. (a) M10 has lost all three exons (3,5 and 9).

6.4 DISCUSSION

Pvu II was found to be much more effective (by a factor of about 11) than Eco RI in the induction of mutations at the *tk* locus. A straight line through the data points has been drawn, however this does not necessarily imply any particular significance to the dose-effect relationship. The background mutations are given in Table 6.1 and have been subtracted from the values shown in figure 6.3. The reason for the relatively high level of mutations induced by electroporation treatment alone (background) is not understood and this background did not appear to correlate with a high chromosomal aberration frequency in electroporated cells (Moses et al,1990). An X-ray dose-effect curve, redrawn from chapter 4 is also included in figure 6.4 for comparison (the top horizontal axis represent dose in Grays). It has been shown for Pvu II at 200 units/ml that dsb are induced over many hours following treatment (see below, Costa and Bryant,1990a). In this respect, treatment with Pvu II may resemble a chronic, low dose-rate treatment with X-rays. Hence a direct comparison of the X-ray curve (acute exposure) with those of RE induced mutations, based on the numbers of induced dsb, is not possible at this stage.

As discussed above, the results in figure 6.3 show that the blunt-ended dsb generated by Pvu II (recognition sequence: CAG*CTG) are much more effective in causing mutations at the *tk* locus than are cohesive type of dsb generated by Eco RI (recognition sequence: G*AATTC). This result is similar to that obtained when using chromosomal aberrations as an end point (Bryant, 1984, Bryant and Christie, 1989) and for cell killing (Bryant, 1985), which supports the notion that the induction of mutations and cell killing might stem from the induction of chromosomal aberrations of deletion (break) or exchange types provided such a change in the DNA sequence gives rise to a non-lethal mutation. This notion is corroborated by results from the derived radiosensitive mutant line *xrs* 5 where a deficiency in dsb repair (Kemp et al, 1984., Costa and Bryant,1988), higher yields of chromosomal aberrations (Kemp and Jeggo,1986., Darroudi and Natarajan,1987b., Bryant et al,1987., Macleod and Bryant,1990), increased mutation frequency (Mussa et al.,1990) and an increased cell killing appear to be intercorrelated.

Molecular cloning and nucleotide sequencing of an approximately full-length Chinese hamster *tk* cDNA has led to the resolution of the hamster *tk* gene (Lewis,1986). There are four and six restriction sites within the *tk* gene for Pvu II and Eco RI respectively (Figure 6.13). The higher mutation frequency observed in Pvu II compared with Eco RI treated cells thus does not result from a difference in the frequency of Pvu II and Eco RI sites in the *tk* gene. The lower number of Pvu II sites in the *tk* gene further accentuates the importance of blunt-ended dsb as critical lesions in mutagenesis although the possibility of a difference in the accessibility of these two enzymes to sites in the *tk* gene; e.g. due to different degrees of condensation of the DNA in different parts of the gene can not be ruled out. It is of interest at this point to note that Hpa I induced mutation frequencies (figure 6.6) are lower than those observed in Pvu II treated cells (figure 6.3) despite the use of higher RE concentrations. Since there are 4 Pvu II restriction sites compared to the single Hpa I site, this would suggest that a relationship exists between the number of sites producing blunt-ended dsb and and the ability to induce mutations. However, this relationship does not hold true for blunt- and cohesive-ended dsb as already discussed above for Pvu II and Eco RI mutation induction/no. of restriction sites.

The importance of blunt-ended dsb with respect to mutation induction is further supported by the increased mutation frequencies observed in cells treated with Hpa I compared to those treated Xho I (figure 6.6), these RE generating blunt- and cohesive-ended dsb respectively. There is one restriction site for each Hpa I and Xho I in the genomic *tk* gene (figure 6.14) separated by approximately 250 bp. This relatively close proximity of the two RE-sites will reduce any positional effects on the induction of mutations as well as render both sites near-similarly accessibe to the RE, a possible factor which has been suggested by some to account for the difference in the effectiveness of various RE to give rise to a variety of end-points (Winegar, 1988).

A possible explanation for the different effectiveness of blunt- and cohesive-ended dsb could be that blunt-ended dsb are repaired at a slower rate than the cohesive type (Bryant,1984). This is indicated in recent studies (Costa and Bryant,1990a,b) in which neutral filter elution was used to measure the kinetics of dsb induced by Pvu II

which showed that dsb accumulated with time for up to 24 hrs in CHO KI cells, suggesting that incision of DNA exceeded the rate of dsb rejoining, whereas for Bam HI and Eco RI (producing cohesive termini), the rate of rejoining was apparently higher than the incision rate so that dsb did not accumulate. The accumulation of dsb could not be attributed to cell death and DNA degradation. Costa and Bryant (1990a) showed this using the trypan blue exclusion assay in which only 0.5% cell death occured at either 0 hrs or 24 hrs after treatment with RE. Also using the DNA precipitability assay, approximately 1% DNA from RE treated cells failed to precipitate in trichloroacetic acid (TCA) both at 0 hrs and 24 hrs after treatment. Results from both these assays thus suggested that accumulation of dsb was indeed due to cutting of DNA by RE rather than DNA degradation as a result of cell death in RE treated cells.

Loss of DNA sequence in the *tk* gene may have resulted from a chromosomal deletion or a rearrangement induced as a result of non-repair or misrepair of blunt-ended dsb which is finally expressed as a viable (non-lethal) mutation. Since the kinetics of Pvu II induced dsb are such that the dsb are produced over many hours following treatment (Costa and Bryant, 1990a,b), it would be premature at this stage to deduce anything from the shape of the enzyme dose-effect curve . However like X-rays, Pvu II induced high frequencies of *tk*- mutants, again confirming our belief that the *tk* locus in CHO KI cells is functionally heterozygous. Evidence of both non-repair and misrepair of dsb is provided by the radiosensitive mutant cell line *xrs* 5 cells which, when exposed to either RE or X-rays, show an increased formation of deletion and exchange types of chromosome aberrations when compared to the wild type CHO KI parent line (Bryant et al, 1987). This increased formation of chromosome aberrations in *xrs* 5 is accompanied by an enhanced mutability at the *tk* (Mussa et al,1990) and at the *hprt* locus (Darroudi and Natarajan,1989) following exposure to X-rays. All the above data provides further evidence for an intercorrelation between dsb, chromosome aberrations and mutation induction.

An earlier study (Obe et al,1986) showed that Alu I (generates blunt-ended dsb) produced mutations at the *hprt* locus which were accompanied by chromosomal aberrations; however, in the same study, Alu I was found to be ineffective in inducing point mutations at

the Na^+/K^+ ATPase locus measured by ouabain resistance. This data was interpreted to mean that most of the Alu I-induced *hprt* mutations occured as a result of a major structural change in the X-chromosome carrying this gene which is lethal at the Ouabain locus. Southern blot analysis show that the majority of radiation-induced mutations at the *hprt* locus in V79 cells (Vrieling et al, 1985., Thacker,1986) are associated with large losses of DNA. This suggests that mutations at these loci are essentially 'chromosomal' mutations, meaning that they involve the loss or rearrangement of large amounts of DNA. Based on the above mentioned data, it is likely that RE-induced mutants similarly occur as a result of chromosomal mutations. This view is supported by the increased production of chromosome aberrations observed in V79 and CHO KI Chinese hamster cells treated with Pvu II (Bryant,1984., Bryant et al,1987) further indicating a common origin (DNA lesion) for both chromosomal aberrations and mutations.

In figure 6.7, we have made a comparison of the frequencies of mutations at both the *tk* and *hprt* loci following X-irradiation (*hprt* data from Darroudi and Natarajan,1989 and Thacker and Cox,1975). The heterozygous *tk* locus in CHO KI cells is clearly mutated at a higher frequency (by a factor of about 3) compared to the hemizygous *hprt* locus in both CHO KI and V79 cells. A higher mutability at the *tk* locus than at the *hprt* locus has also been observed in the mouse lymphoma L5178Y cell line and this is thought to result in the case of *hprt* due to its poor recovery from damage to flanking essential genes on the single copy X-chromosome (Evans et al,1986). A similar comparison between data for mutations at the *tk* and *hprt* loci can be made for cells treated with restriction endonucleases (figure 6.8). In this case, Pvu II can be seen to induce higher frequencies of mutations at the *tk* locus (present work) than those induced by Alu I (inducing dsb with blunt termini) at the *hprt* locus (data from Obe et al,1986) in V79 cells. Like X-rays, Pvu II induced mutations at the *tk* locus show a similar higher mutation frequency (also by approximately a factor of 3) over that observed at the *hprt* locus following treatment with Alu I. This not only further exemplifies the sensitivity of the *tk* locus to DNA damage but also provides additional evidence that blunt-ended dsb induced by Pvu II truly mimic radiation-induced pre-mutational lesions are thus representative of the type of initial damage (i.e dsb) which is

misrepaired, fixed and expressed as a viable mutation in X-irradiated cells. However, an exact comparison of the Alu I mutation data (Obe et al,1986) with Pvu II induced mutation frequencies cannot be made because of differences in the method of treating cells with the RE. Moreover, it is possible that Alu I cuts the *hprt* gene at a different frequency from that of Pvu II at the *tk* locus. Similar to the above comparison, a higher mutation induction is observed at the *tk* locus than at the *hprt* locus in Pvu II treated cells (figure 6.12). In this case, unlike the Alu I comparison made above, both the cell lines were exposed to similar conditions hence this result would provide further support for a similarity to the X-ray induced curves (figure 6.7), further suggesting the importance of blunt-ended dsb in representing radiation-induced pre-mutational lesions.

Analysis of molecular changes in the DNA of mutant cells is essential for a better understanding of the process of mutagenesis. This goal has been achieved recently with the isolation of mutants at the *aprt* gene in hamster cells which due to its small size (< 4 kb) has been well sequenced in many mutant cells thus allowing the identification of small changes such as point mutations (Nalbantoglu et al, 1987). Similar analyses have been achieved with the *hprt* gene following treatment with various types of ionising radiation (Brown and Thacker, 1984., Brown et al, 1986., Thacker, 1986., Stankowski et al, 1986., Fuscoe et al, 1986). The isolation of *hprt*- mutants in V79 cells following treatment with Pvu II, a RE which simulates blunt-ended dsb was the first step in looking at the molecular changes associated with 'pure' dsb without the interference of other lesions, as in the case of treatment following ionising radiation. The DNA of the *hprt* mutant cells was isolated and using two RE namely Eco RI and Pst I, the restriction fragment patterns on the southern blots were compared with a map of the parent V79 hamster *hprt* gene (Rossiter, 1987). In figure 6.15, following treatment with Eco RI, the mutant M2 has lost a 11 kb fragment indicating a possible deletion at the 5' end of the gene while the mutant M10 has lost both gene fragments. The loss of the two large Eco RI fragments adds up to >20 kb which probably contains the whole functional gene which would suggest the existence of a large deletion resulting in loss of whole of the functional gene. This is supported by the Pst I restriction fragment pattern in which M2 shows a slight alteration in the largest (faint) fragment which would

also suggest a 5' alteration (not definite). In the same blot, M10 shows only a pseudogene fragment further suggesting a complete gene loss. In figure 6.16, the Eco RI restriction pattern shows M7 to have lost all of the gene fragments which is confirmed in the Pst I blot where M7 is the only definite deletion mutation (only the pseudogene fragment is visible). In addition to this, the largest fragment of M8 seems to be missing which would suggest an alteration at the 5' end of the gene in this mutant. Finally, in figure 6.17, M12 has a complete deletion with all the fragments missing in the Eco RI restriction pattern. This is supported by the loss of all the band except for a pseudogene fragment in the Pst I restriction pattern. The remaining mutants (10/15), which show no changes in the southern blots probably have small mutations beyond the limits of detection of Southern blot analysis, or mutations in regions which are not covered by the probe. In addition, it is possible that the mutants with an unaltered gene fragment pattern are of spontanoeus origin (Brown and Thacker, 1984) although this is highly unlikely in view of the low frequency of spontaneously induced *hprt-* mutants (Table 6.2). Hence, this preliminary data suggests that 5/15 Pvu II-induced mutants show large deletions some of which are associated with the loss of the whole of the functional *hprt* gene.

In Southern analysis, one band often contains more than one exon of the *hprt* gene (Rossiter et al, 1991) which reduces the resolution in determining the extent of a deletion. The availability of the primer sequence for the 9 exons in the *hprt* gene has enabled the use of PCR to amplify specific exons to confirm and further resolve the results obtained with Southern analysis. For the present experiments, exon 3, 5 and 9 were amplified in all the mutants (M1-M15). M2 has lost exon 3 while 5 and 9 are present. This would suggest a deletion at the 5' end confirming southern blot analysis (figure 6.18-6.20). M7, M10 and M12 has lost all exons 3, 5 and 9 confirming the presence of a large deletion with the loss of the whole gene. All the other mutants 11/15 do not contain deletions in the regions of exons 3, 5 or 9. However, this does not rule out changes in other parts of the gene especially the 5' end, which has not been analysed due to the difficulty in getting primers for exon 1 to work.

The presence of the large deletion mutants observed following treatment with RE is similar to in deletion-mutants induced following treatment with ionising radiation but at a lower frequency.

Treatment	Number of mutants analysed	Large changes (deletions)
Pvu II	15	33%
Ionising radiation	30-50	70% *

Table 6.3. The percentage of mutants (*hprt*-) which show large deletions following treatment with RE and ionising radiation.
(* Data from Brown and Thacker, 1984., Brown et al, 1986, Thacker, 1986).

Extensive studies of mutations at the *hprt* gene in hamster cells have found that large deletions occur in about 70% of mutants induced by ionising radiation (Thacker, 1986., Stankowski et al, 1986., Fuscoe et al, 1986). The number of RE-induced mutants analysed is too small to make a definite conclusion but this preliminary data would support a relationship between blunt-ended dsb and the induction of large deletions. The above results would provide a plausible explanation for the increased mutation frequencies at the *tk* locus observed in CHO KI cells treated with both Pvu II and Hpa I, RE which generate blunt-ended dsb.

Radiation is thought to induce a mixture of both blunt and cohesive-ended dsb with a predominance of the latter type and this was suggested as a possible reason for the relatively low efficiency of the conversion of X-ray induced dsb into chromosomal aberrations (Bryant, 1990). Despite the fact that the 'clean' breaks (those with 3' hydroxyl and 5' phosphoryl termini) induced by RE are unlikely to be generated by X-rays, the large deletions observed in RE-induced *hprt* mutants, though preliminary, would point to dsb as critical lesions in mutagenesis due to misrepair as a result of the reduced repair rate (Costa and Bryant,1990 a,b). This approach of using RE to mimic

radiation-induced mutagenesis offers a model system which may lead to a future understanding of the mode of conversion of X-ray induced dsb into mutations for elucidating the molecular nature of mechanism(s) for the formation of mutations. Gibbs et al (1987) suggested dsb as the principle mutagenic lesion induced by X-rays. This notion is supported by the present investigation which in addition suggests that mutations caused by X-rays are likely to arise principally via dsb with blunt-ended termini.

CONCLUSIONS

The results obtained during the course of this project, show the the autosomal *tk* locus in Chinese hamster (CHO KI) cells as a highly sensitive locus. This allowed the isolation and measurement of *tk*- mutants at a very high frequency following treatment with X-rays or RE. The reason for this high sensitivity to mutagens may be that the *tk* locus resides on a chromosome with non-essential flanking regions so that a deletion of a large part of this chromosome does not result in death of the mutant cells. This view is supported by more recent studies which suggest that the heterozygous nature of the *tk* locus permits the recovery of both single-gene and large chromosomal mutations, while the more commonly used *hprt* permits the recovery of mutations affecting only single gene only (Evans et al, 1986., Moore et al, 1987, 1989). Based on the results of the subsequent analysis of the induced *tk*- mutants, the stability of these mutants indicates that the mutation system selects for 'true' stable *tk*- mutants rather than an unstable mutational change.

The increased frequency of mutations observed in the *xrs 5* cell line compared to the parent CHO KI cells line (Chapter 4) provides evidence for the importance of DNA repair and more specifically dsb repair in preventing the induction of a mutation.

This view is supported by the increased mutation frequency observed in CHO KI cells treated to X-rays in the presence of a DNA synthesis inhibitor (ara A), which is also known to inhibit dsb repair. This result helps to understand the hypermutability observed in xrs 5 cells, and is further evidence of the importance of dsb in giving rise to mutations. In addition to this, the increased mutation frequency in the presence of ara A points to the existence of a sub-class of dsb, which are repaired independently of DNA polymerization, possibly via a simple ligation step. Since the ligation step does usually not require any exonuclease activity, this class of dsb may be more prone to misrepair/misjoining, thus accounting for the increased induction of mutations.

The high frequency of chromatid breaks in ara A treated cells (the experimental schedule for treatment with ara A was similar for both mutation and G_2 assay) provides indirect evidence for dsb repair inhibition by ara A. This correlation between the increased mutation induction and chromatid breaks, may suggest a similar mechanism by which inhibition of the dsb repair leads to the induction of mutations

and formation of chromatid breaks, the former being non-lethal while the latter may be lethal. This correlation is further supported by the increased frequency of chromosome aberrations observed in Chinese hamster cells exposed to the similar conditions used in the mutation experiments e.g. X-rays and RE (Bryant et al, 1987., Darroudi and Natarajan, 1987a,b).

The increased frequency of mutations observed in cells treated with RE generating blunt-ended dsb compared to cohesive-ended dsb, suggests further a sub-class of dsb (on the basis of the end-structure of dsb) with respect to the ability to induce mutations. An increased frequency of misrepair of blunt-ended dsb, possibly due to the destabilized covalent bonding at point of breakage may be responsible for giving rise to the increased induction of mutations. This is contrary to cohesive-ended dsb, which due to the presence of intervening hydrogen-bonds near point of breakage, may hold dsb-ends together thus making the damaged site more accessible to the repair enzymes. This in turn will increase the fidelity of repair of this type of dsb thus accounting for the reduced induction of mutations. A simple ligation repair has been suggested to exist for blunt-ended dsb (Costa, 1990c). This type of repair as suggested for the ara A data above, may explain the increased effectiveness of blunt-ended dsb in inducing mutations.

Blunt-ended dsb (induced by Pvu II) were found to generate mutations at the *hprt* locus and subsequent molecular analysis (southern bloting and PCR) of changes associated at this locus showed ~34% to have large deletions. This frequency of deletion mutants is lower than that observed in mutant cells induced following treatment with ionising radiation (60-70%; Thacker et al, 1986). This comparison may be indicative of the difference between radiation and RE-induced dsb in the ability to induced mutations. This study was based on the analysis of only 15 mutants therefore it may be necessary to isolate more RE-induced mutants to provide more conclusive evidence for the possible role of blunt-ended dsb in the induction of large deletions as observed in radiation-induced mutants.

Finally it can be said that this study has provided evidence for the importance of dsb as a pre-mutational lesion and further possible sub-classification of these dsb into the effectiveness to induce mutations; although the exact nature of these critical lesions remains controversial. Despite the fact that 'clean' breaks, those with 3'

hydroxyl and 5' phosphoryl termini induced by RE, are unlikely to be generated by X-rays at a high frequency, the approach used in this thesis of using RE to mimic radiation offers a model system which may lead to a further understanding of the mode of conversion of X-ray induced dsb into mutations.

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APPENDIX

Published articles:

(a). **Singh, B. and P.E. Bryant (1991)** Induction of mutations at the thymidine kinase locus in CHO cells by restriction endonucleases., *Mutagenesis.*, **Vol. 6, No. 3**, 219-223.

(b). **Singh, B. and P.E. Bryant (1990)** Increased mutation induction in Chinese hamster ovary cells exposed to X-rays and ara A., *International. J. Radiation Biology.*, **58**, 900-901.

(c). **Singh, B. and P.E. Bryant (1991)** DNA double-strand breaks as mutagenic lesions., *Proceedings of the 9th International Congress of Radiation Research, (Toronto)* **Vol 1**, pp. 337

(d) **Mussa, T.A.K., Singh, B and P.E. Bryant (1990)** Enhanced mutability at the *tk* locus in the radiosensitive double-strand break repair mutant *xrs* 5., *Mutation Research.*, **231**, 187-193.

(e). **Mussa, T.A.K., Singh, B and P.E. Bryant (1990)** Enhanced mutation frequency at the *tk* locus in the Chinese hamster ovary mutant *xrs* 5., *International. J. Radiation Biology.*, **57**, 1263.

Mutagenesis vol.6 no.3 pp.219–223, 1991

Induction of mutations at the thymidine kinase locus in CHO cells by restriction endonucleases

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Induced mutation frequencies were measured at the *tk* locus (encoding for the enzyme thymidine kinase) following treatment of Chinese hamster ovary cells (CHO KI) with two restriction endonucleases (REs), *Pvu*II and *Eco*RI, which generate 'blunt-ended' and 'cohesive-ended' DNA double-strand breaks (dsb), respectively. Electroporation was used to introduce these enzymes into the cells. Restriction endonucleases generating blunt-ended dsb have been shown to mimic the action of ionising radiation in causing chromosome aberrations, cell killing, mutations and oncogenic transformation. Here we show that at the *tk* locus, *Pvu*II induced an ~11-fold higher mutation frequency than *Eco*RI at the same enzyme concentrations. There are four *Pvu*II and six *Eco*RI restriction sites in the Chinese hamster thymidine kinase gene. Hence the higher mutation induction by *Pvu*II, despite the lower number of restriction sites than *Eco*RI in the *tk* gene, suggests that blunt-ended dsb represent more effective and critical mutagenic DNA lesions than the cohesive-ended type. In this respect, our results are similar to those we obtained previously for chromosomal aberrations and for cell killing. Results from the present study suggest that mutations could arise from unrepaired or misrepaired dsb possibly via induction of chromosomal deletions or stable exchanges between chromosomes.

Introduction

Ionizing radiation has been known to induce a number of lesions in DNA by absorption of energy within or close to the DNA. These include direct-strand breakage, base damage, and the less frequent DNA-protein and DNA–DNA crosslinks. Most of this damage may be repaired by the cellular repair systems while the unrepaired or misrepaired damage may appear in the form of a viable mutation, chromosomal aberrations or even result in cell death. The DNA double-strand break (dsb) appears to be the most important lesion leading to the observed cellular effects of ionizing radiation. Molecular analysis of mutants induced by ionizing radiation showed that a substantial portion (80%) of mutants arise as a result of gross structural damage to the DNA resulting in deletions of large sections of essential DNA sequence (Vrieling *et al.*, 1985; Thacker, 1986; Yandell *et al.*, 1986; Liber *et al.*, 1987). Cytogenetic analysis of cells exposed to ionizing radiation shows a dose-related formation of chromosome aberrations which have been suggested to arise due to misrepair or to failure of the repair system to remove the induced dsb produced by ionizing radiation (Natarajan *et al.*, 1980).

We have previously suggested that the Chinese hamster (CHO KI) line in our laboratory is functionally heterozygous with respect to the *tk* locus and therefore mutates at a very high frequency, providing a highly sensitive locus for the assay of mutation (Mussa *et al.*, 1990). The importance of dsb in the induction of mutations by radiation is indicated by our previous work (Mussa *et al.*, 1990) with the X-ray sensitive (*xrs* 5) cell line, a mutant line deficient in dsb repair (Jeggo and Kemp *et al.*, 1983; Kemp *et al.*, 1984). This line was found to be hypermutable compared to its parent cell line (CHO KI) following treatment with X-rays. In addition, treatment of permeabilized Chinese hamster cells with restriction endonucleases (REs) has been shown to induce mutations at the *hprt* locus (Obe *et al.*, 1986). REs have the property to recognize and cut the DNA double-helix at specific sites. This property allows REs to be used in order to mimic radiation not only in inducing mutations but also causing chromosomal aberrations, cell killing and oncogenic transformation (Bryant, 1984, 1985; Bryant and Riches, 1989).

In the present study, we have further investigated the relationship between dsb and mutations by treating cells with REs and measuring mutations at the thymidine kinase (*tk*) locus in CHO K1 cells. REs were used that cause dsb with either blunt- or cohesive-ended termini (*Pvu*II and *Eco*RI, respectively), and the yield of mutations at the *tk* locus assayed by plating cells in trifluorothymidine (TFT) selection medium. We have previously shown (Bryant, 1984) that dsb with blunt termini are much more effective than those with cohesive termini in inducing chromosomal aberrations.

Electroporation was used to facilitate the entry of REs into Chinese hamster cells. This method has previously been used to introduce REs into the cells to generate chromosome aberrations (Winegar *et al.*, 1989; Moses *et al.*, 1990). The principle of this technique is based on the discharge of a capacitor through a cell suspension. This causes a localized breakdown of the plasma membrane creating 'pores' (Knight, 1981) and thus allowing cellular uptake of large molecules (Chu *et al.*, 1987) such as REs.

Materials and methods

Cell culture

Chinese hamster ovary (CHO K1) cells were used. Cells were routinely maintained in Eagle's minimum essential medium (MEM, Gibco) supplemented with fetal calf serum (10% v/v) and non-essential amino acids. Experiments were performed with an asynchronous population of exponentially growing cells.

Purification of enzymes

The REs *Pvu*II and *Eco*RI (Northumbria Biologicals Ltd, UK) were purified using Amicon 10 ultrafilters to remove the storage buffers (Bryant and Christie, 1989). The purified enzymes were then diluted to 10 U/μl in calcium-free Hanks balanced salt solution (HBSS) containing 6 mmol/l $MgCl_2$ and 1% bovine serum albumin (HBSS-BSA). Both *Pvu*II and *Eco*RI were found to be fully active after purification and during incubation for at least 9 h in HBSS-BSA at 37°C. This was verified using an *in vitro* plasmid (pBR322) digestion assay (unpublished data). At the present stage, it is not possible to determine directly the cutting activity of RE within the cellular environment because both cutting and repair of RE induced dsb are thought to occur simultaneously (Costa and Bryant, 1990a).

Electroporation

Exponentially growing cells were trypsinized from culture flasks and suspended in HBSS-BSA. Cells were centrifuged and resuspended in HBSS-BSA twice. The final cell concentration was adjusted to 10^6 cells/ml in HBSS-BSA. Electropora-

tion was performed using a Cell-Porator™ (Bethesda Research Laboratories). Purified RE (*Pvu*II and *Eco*RI; 5–30 U) was mixed with 1 ml of cell suspension (10^6 cells) in an Eppendorf tube before pipetting into a disposable electroporation chamber. The Cell-Porator™ was set to the following parameters: field strength, 750 V/cm; capacitance, 1600 μF; resistance, low; temperature, ambient. The electroporation chamber was rinsed with HBSS-BSA between each enzyme treatment.

Immediately after electroporation, samples were poured into 15 ml conical centrifuge tubes containing 5 ml of warm MEM medium. Samples in medium were then centrifuged and the supernatent aspirated. Cells were resuspended in 10 ml of fresh medium, transferred into 75 cm^2 flasks (Sterlin) and incubated for 4 days (Mussa *et al.*, 1990) at 37°C. The medium was changed after 24 h. All electroporations were repeated twice for each dose point.

Mutation assay

After 4 days expression time, cells were trypsinized and the suspension diluted to give ~ 10^6 cells/dish in 9 cm tissue culture grade Petri dishes (Sterlin) with 9 ml of MEM/trifluorothymidine (TFT, Sigma) at a concentration of 3 μg/ml TFT (Adair and Carver, 1979). Dishes were incubated in a humidified incubator at 37°C with 5% CO_2 in air for 12 days. Following aspiration of medium, colonies were fixed with methanol and stained with giemsa prior to scoring. Cells were also diluted to ~ 100–200 cells per dish (5 cm) and incubated for 8 days in non-selective medium (MEM) to assay for the number of viable cells in the culture after the 4 days expression period. The total number of colonies in TFT plates and those in non-selective medium were counted, and the counts used to calculate the tk^- mutation frequency per viable cell.

Results and discussion

Results for mutations induced at the *tk* locus in CHO KI cells by treatment with the REs *Pvu*II and *Eco*RI are shown in Figure 1. The frequency of induced mutations was enzyme-dose dependent for both enzymes; however, *Pvu*II was found to be much more effective (factor of ~ 11) than *Eco*RI in the induction of mutations at the *tk* locus. We have drawn a straight line through the data points; however, by doing this we do not mean to attach any particular significance to the dose–effect relationship. The background mutations are given in Table I and have been subtracted from the values shown in Figure I. The reason for the relatively high levels of mutations induced by electroporation treatment alone (background) is not understood and this background did not appear to correlate with a high chromosomal aberration frequency in electroporated cells (Moses *et al.*, 1990). An X-ray dose–effect curve, redrawn from Mussa *et al.* (1990), is also included in Figure 1 for comparison (the top horizontal axis represent dose in Grays). It has been shown that for *Pvu*II, the dsb are induced over many hours following treatment (see below, Costa and Bryant, 1990a). In this respect, treatment with *Pvu*II may resemble a chronic, low dose-rate treatment with X-rays. Hence a direct comparison of the X-ray curve (acute exposure) and those of RE-induced mutations, based on the numbers of induced dsb, is at this stage not possible.

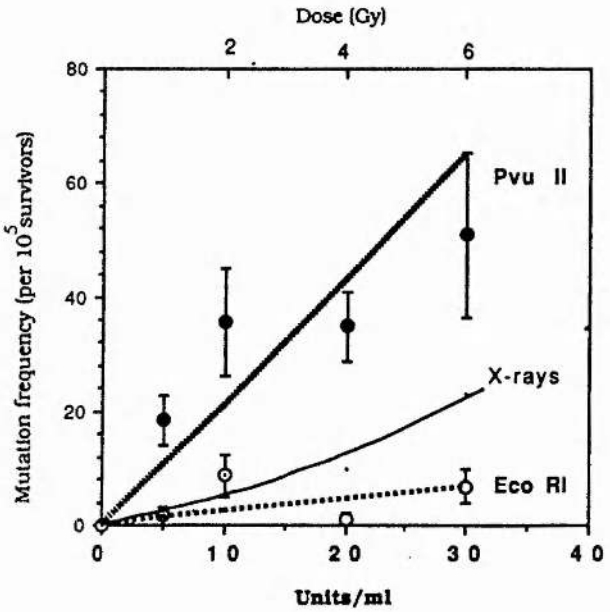

Fig. 1. Induced mutation frequencies at the *tk* locus in CHO cells as a function of the concentration of *Pvu*II and *Eco*RI. A curve of X-ray induced mutations is also shown (redrawn from Mussa *et al.*, 1990). All lines are fitted by eye and vertical bars represent standard errors of mean values from three experiments.

As mentioned above, results in Figure 1 show that the blunt-ended dsb generated by *Pvu*II (recognition sequence: CAG*CTG) are much more effective in causing mutations at the *tk* locus than are cohesive type of dsb generated by *Eco*RI (recognition sequence: G*AATTC). This result is similar to that obtained when using chromosomal aberrations as an end point (Bryant, 1984; Bryant and Christie, 1989) and for cell killing (Bryant, 1985), which supports the notion that induction of mutations and cell killing might stem from the induction of chromosomal aberrations of deletion (break) or exchange types provided such a change in the DNA sequence gives rise to a non-lethal mutation. This notion is corroborated by results from the derived radiosensitive mutant line *xrs*5 where a deficiency in dsb repair (Kemp *et al.*, 1984; Costa and Bryant, 1988), higher yields of chromosomal aberrations (Kemp and Jeggo, 1986; Darroudi and Natarajan, 1987; Bryant *et al.*, 1987; Macleod and Bryant, 1990), increased mutation frequency (Mussa *et al.*, 1990) and an increased cell killing appear to be intercorrelated. The reason for the lower efficiency of dsb with cohesive termini in causing mutations or chromosomal aberrations is not yet fully understood but may have to do with the more rapid and efficient repair of cohesive-ended dsb as compared with blunt-ended dsb (Bryant, 1988; Costa and Bryant, 1990a,b).

Molecular cloning and nucleotide sequencing of an approximately full-length Chinese hamster *tk* cDNA has led to the resolution of the hamster *tk* gene (Lewis, 1986). There are four and six restriction sites within the *tk* gene for *Pvu*II and *Eco* RI, respectively (Figure 2). The higher mutation frequency observed in *Pvu*II compared with *Eco*RI treated cells thus does not result

Table I. Frequency of TFT-resistant mutations in untreated and electroporated samples

Sample	Mutations per 10^5 survivors[a]
Untreated cells	3.6
Electroporated cells	19.3

[a]Average of three independent experiments

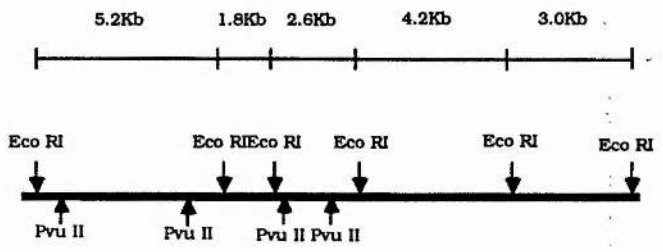

Fig. 2. A restriction map of the Chinese hamster *tk* gene showing both *Pvu*II and *Eco*RI sites. Size of *Eco*RI restriction fragments are also shown (redrawn from Lewis, 1986).

from a difference in the frequency of *Pvu*II and *Eco*RI sites in the *tk* gene. The lower number of *Pvu*II sites in the *tk* gene further accentuates the importance of blunt-ended dsb as critical lesions in mutagenesis, although we cannot rule out the possibility of a difference in the accessibility of these two enzymes to sites in the *tk* gene, e.g. due to different degrees of condensation of the DNA in different parts of the gene. A possible explanation for the different effectiveness of blunt- and cohesive-ended dsb could be that blunt-ended dsb are repaired at a slower rate than the cohesive type (Bryant, 1984). This is indicated in recent studies

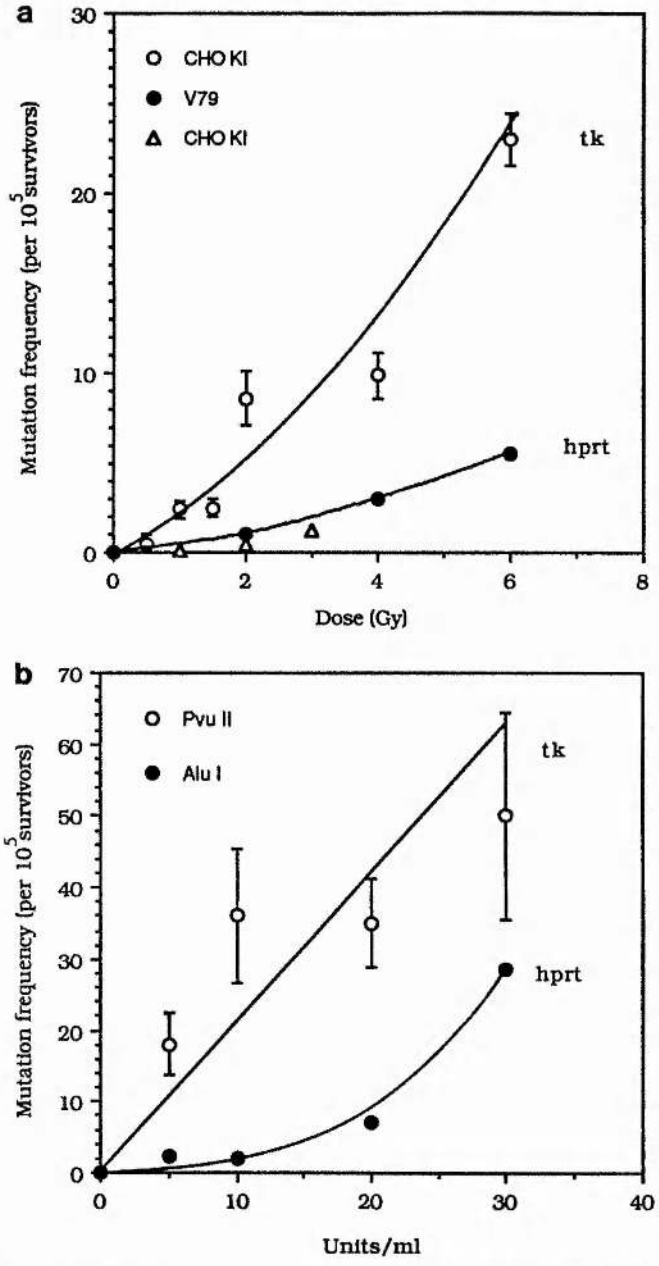

Fig. 3. (a) X-ray induced mutations at the *tk* and hprt loci [(V79/*hprt* data from Van Zeeland and Simons (1976) and CHO/*hprt* data from Darroudi and Natarajan, (1989)]. Vertical bars represent standard errors of mean values from three experiments. (b) Induced mutation frequencies at the *tk* locus in CHO cells exposed to *Pvu*II and at the *hprt* locus in V79 cells exposed to *Alu*I [data from Obe *et al.* (1986)]. Vertical bars represent the standard errors of mean values from three experiments.

(Costa and Bryant, 1990a,b) in which neutral filter elution was used to measure the kinetics of dsb induced by *Pvu*II which showed that dsb accumulate with time up to 24 h in CHO KI cells, suggesting that incision of DNA exceeded the rate of dsb rejoining whereas for *Bam*HI and *Eco*RI (producing cohesive termini), the rate of rejoining was apparently higher than the incision rate so that dsb did not accumulate. The accumulation of dsb could not be attributed to cell death and DNA degradation. Costa and Bryant (1990a) showed this using the trypan blue exclusion assay in which only 0.5% cell death occurred at either 0 or 24 h after treatment with RE. Also, using the DNA precipitability assay, ~ 1% DNA from RE-treated cells failed to precipitate in trichloroacetic acid (TCA) both at 0 and 24 h after treatment. Results from both these assays thus suggested that accumulation of dsb was indeed due to cutting of DNA by RE rather than DNA degradation as a result of cell death in RE-treated cells.

Loss of DNA sequence in the *tk* gene may have resulted from a chromosomal deletion or a rearrangement induced as a result of non-repair or misrepair of blunt-ended dsb and which is finally expressed as a viable (non-lethal) mutation. Since the kinetics of *Pvu*II-induced dsb are such that the dsb are, as mentioned above, produced over many hours following treatment, it would be premature at this stage to deduce anything from the shape of the enzyme dose–effect curve. However, like X-rays, *Pvu*II-induced high frequencies of tk^- mutants, again confirming our belief that the *tk* locus in CHO KI cells is functionally heterozygous (Mussa *et al.*, 1990). Evidence of both non-repair and misrepair of dsb is provided by the radiosensitive mutant cell line *xrs*5 cells which when exposed to either REs or X-rays shows an increased formation of deletion and exchange type of chromosome aberrations when compared to the wild-type CHO KI parent line (Bryant *et al.*, 1987). This increased formation of chromosome aberrations in *xrs*5 is accompanied by an enhanced mutability at the *tk* (Mussa *et al.*, 1990) and at the *hprt* locus (Darroudi and Natarajan, 1989) following exposure to X-rays. All the above data provides evidence for an intercorrelation between dsb, chromosome aberrations and mutation induction.

An earlier study, Obe *et al.* (1986) showed that REs produced mutations at the *hprt* locus which were accompanied by chromosomal aberrations; however, REs were found to be ineffective in inducing point mutations at the Na^+/K^+ ATPase locus measured by ouabain resistance. This data was interpreted to mean that most of the RE-induced *hprt* mutations occur as a result of a major structural change in the X-chromosome carrying this gene. Southern blot analysis shows that the majority of radiation-induced mutations at the *hprt* locus in V79 cells (Vrieling *et al.*, 1985; Thacker, 1986) are associated with large losses of DNA. This suggests that mutations at these loci are essentially 'chromosomal' mutations, meaning that they involve the loss or rearrangement of large amounts of DNA. Based on the above mentioned data, it is likely that RE-induced mutants similarly occur as a result of chromosomal mutations. This view is supported by the increased production of chromosome aberrations observed in V79 and CHO KI Chinese hamster cells treated with *Pvu*II (Bryant, 1984; Bryant *et al.*, 1987) further indicating a common origin (DNA lesion) for both chromosomal aberrations and mutations.

In Figure 3a, we have made a comparison of the frequencies of mutations at both the *tk* and *hprt* loci following X-irradiation [*hprt* data from Darroudi and Natarajan (1989) and Van Zeeland and Simons (1976)]. The heterozygous *tk* locus in CHO KI cells

is clearly mutated at a higher frequency (factor of ~ 3) compared to the hemizygous *hprt* locus in both CHO KI and V79 cells. A higher mutability at the *tk* locus than at the *hprt* locus has also been observed in the mouse lymphoma L5178Y cell line and this is thought to result in the case of *hprt*, from poor recovery from damage to flanking essential genes on the single copy X-chromosome (Evans *et al.*, 1986). A similar comparison between data for mutations at the *tk* and *hprt* loci can be made for cells treated with restriction endonucleases (Figure 3b). In this case, *Pvu*II can be seen to induce higher frequencies of mutations at the *tk* locus (present work) than those induced by *Alu*I (inducing dsb with blunt termini) at the *hprt* locus (data from Obe *et al.*, 1986) in V79 cells. Like X-rays, *Pvu*II-induced mutations at the *tk* locus show a similar higher mutation frequency (also a factor of ~3) over that observed at the *hprt* locus following treatment with *Alu*I. This not only further exemplifies the sensitivity of the *tk* locus to DNA damage but also provides additional evidence that blunt-ended dsb induced by *Pvu*II truly mimic radiation-induced pre-mutational lesions are thus representative of the type of initial damage (i.e. dsb) which is fixed and expressed as a viable mutation in X-irradiated cells. However, an exact comparison of the *Alu*I mutation data (Obe *et al.*, 1986) with *Pvu*II-induced mutation frequencies cannot be made because of differences in the method of treating cells with the RE. Moreover, it is possible that *Alu*I cuts the *hprt* gene at a different frequency from that by *Pvu*II at the *tk* locus.

Radiation is thought to induce a mixture of both blunt- and cohesive-ended dsb with a predominance of the latter type, and this was suggested as a possible reason for the relatively low efficiency of the conversion of X-ray-induced dsb into chromosomal aberrations (Bryant, 1989). Despite the fact that the 'clean' breaks (those with 3′ hydroxyl and 5′ phosphoryl termini) induced by RE are unlikely to be generated by X-rays, the approach of using RE to mimic radiation offers a model system which may lead to a future understanding of the mode of conversion of X-ray induced dsb into mutations. Gibbs *et al.* (1984) suggested dsb as the principle mutagenic lesion induced by X-rays. This notion is supported by the present investigation which in addition suggests that mutations caused by X-rays are likely to arise principally via dsb with the blunt-ended termini.

Acknowledgements

We would like to thank Mr J.McIntyre for his technical support. B.S. is supported by a grant from British Nuclear Fuels plc. The work was also supported by funds from the Cancer Research Campaign.

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Induced mutation frequencies at the thymidine kinase (tk) locus were measured in Chinese hamster ovary cells (CHO KI). Confluent cell cultures were exposed to X-rays alone or in combination with 9-ß-D-arabinofuranosyladenine (Ara A) was used at a concentration of 100 μM and incubated with cell cultures for 3 hr postirradiation. Selection of the mutant cells (tk-) was carried out after a expression period of 4 days by plating cells in medium containing trifuorothymidine (TFT, 3μg/ml). Induced mutation frequencies were calculated after 12 days growth of colonies on TFT plates. Previous studies have shown ara A to be an inhibitor of DNA double-strand break (dsb) resulting in increased cell killing (Iliakis, Radiation Research, 83, 537, 1980) and chromosomal aberrations (Bryant, International Journal of Radiation Research, 43, 459, 1983). Results of the present study suggest that unrepaired or misrepaired dsb represent critical lesions in the expression of mutation in irradiated cells.

Mutation Research, 231 (1990) 187–193
Elsevier

MUT 04881

Enhanced mutability at the tk locus in the radiosensitive double-strand break repair mutant *xrs5*

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(Received 23 October 1989)
(Revision received 13 February 1990)
(Accepted 12 March 1990)

Keywords: Mutation frequency; Expression time; DNA double-strand break; Xrs5 mutant cell line; CHO K1 cell line; Thymidine kinase locus

Summary

The thymidine kinase locus (tk) has been utilised as the target locus to measure the induced mutation frequency following X-irradiation in the X-ray-sensitive *xrs5* mutant and its parent CHO K1 line of Chinese hamster cells. Mutations to tk^- cells were measured by plating cells in selective medium containing trifluorothymidine after a post-irradiation expression time of 4 days. Our results show that the mutation frequency was 3–4 times higher in the *xrs5* mutant than in the CHO K1 cell line. This enhanced mutation frequency in *xrs5* is though to result from the deficiency in DNA double-strand break repair in this cell line which also results in the enhanced cell killing and higher frequencies of chromosomal aberrations in response to X-irradiation. The findings of the present study suggest that DNA double-strand break is a critical lesion leading to mutations in irradiated cells.

Ionising radiation is known to induce a variety of lesions in the DNA of cells. These include direct strand breakage, base damage and less frequent DNA–DNA and DNA–protein crosslinks. Cellular systems may repair some of these lesions while the unrepaired or misrepaired damage may appear in the form of a viable mutation or even result in cell death. Molecular analysis of mutants induced by ionising radiation suggested that a substantial portion (80%) of mutants occur as a result of gross structural damage to the DNA resulting in deletions of large sections of the sequence (Kavathas et al., 1980; Orr et al., 1982; Graf and Chasin, 1982; Thacker, 1986; Yandell et al., 1986; Liber et al., 1987). These may have resulted from DNA double-strand breaks (dsb) since experiments show that both chromosomal aberrations and mutations can be induced by treating cells with restriction endonucleases (Bryant, 1984; Obe et al., 1986).

Some radiosensitive mammalian lines show an enhanced mutational response (e.g. Evans et al., 1985) which has been attributed to the lack of a repair process (Evans et al., 1986). A notable

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exception to this is ataxia-telangiectasia (A-T) cells which are sensitive to ionising radiation (Taylor et al., 1975) but are not found to be hypermutable (Arlett and Harcourt, 1978; Arlett, 1980). However A-T cells have not been found deficient in the repair of dsb (Lehmann and Stevens, 1977), although the fidelity of this repair may be lower than in normal cells (Cox et al., 1986; Debenham et al., 1987).

Using the X-ray-sensitive *xrs5* mutant of the CHO K1 cell line (Jeggo and Kemp, 1983) which has been shown to be deficient in dsb repair (Kemp et al., 1986; Costa and Bryant, 1988) we have addressed the question of whether the deficiency in the repair of dsb leads to a higher mutation frequency or whether the enhanced lethality would override the mutational events thereby leading to a reduced mutation frequency. An enhanced mutation frequency in repair-deficient cells may depend critically on the balance between the cell killing and mutagenic potential of the induced lesion. In our investigation, we have utilised the autosomally located thymidine kinase locus (tk) as the target locus to compare the mutational response in the radiosensitive mutant line *xrs5* and its parent Chinese hamster ovary line (CHO K1). The tk locus which has been widely used in previous studies in various cell lines (Clive et al., 1979; Hozier et al., 1981; Evans et al., 1986; Yandell et al., 1986) appears to be heterozygous in the CHO K1 cells thus providing a sensitive selection system when using trifluorothymidine (TFT) as the selective agent. Previous investigators have successfully selected thymidine kinase mutants (tk^-) using TFT as the selective agent (Brown and Clive, 1978; Adair and Carver, 1979; Moore et al., 1989).

Materials and methods

Cell culture

The X-ray-sensitive *xrs5* line of Jeggo and Kemp (1983) and its wild-type parent line CHO K1 were used. These were routinely maintained in Eagle's minimum essential medium (MEM, Gibco) supplemented with fetal calf serum (10%) and non-essential amino acids. Experiments were performed with asynchronous populations of exponentially growing cells.

Mutation assay

Cells were seeded in 75-cm^2 flasks (Sterlin) in culture medium (MEM) at densities ranging from 2×10^5–10^6 cells per flask. Flasks were gassed and incubated at 37 °C for 4 h to allow formation of a cellular monolayer prior to irradiation. Following X-ray exposure, flasks were incubated for 4 days (expression time) to yield the optimum mutation frequencies. After the expression period, cells were trypsinised and the suspension diluted to give approximately 3×10^5–10^6 cells/dish in 9-cm tissue culture grade petri dishes (Sterilin) with 9 ml of MEM/trifluorothymidine (TFT, Sigma) at a concentration of 3 μg/ml TFT (Adair and Carver, 1979). Dishes were incubated in a humidified incubator at 37 °C with 5% CO_2 in air for 12 days. Mutant colonies were fixed with methanol and stained with Giemsa prior to scoring. After irradiation, expression period and trypsinization, survival was assayed by diluting cell suspensions to give approx. 100–200 viable cells/dish (5 cm diameter, Sterilin) in 5 ml MEM. After 7 days incubation at 37 °C, colonies were fixed and stained with Giemsa prior to scoring.

The total numbers of colonies in TFT plates and those in non-selective medium were counted to give an estimate of tk^- mutant frequency. The colonies observed were not found to be of two distinct sizes as has been reported previously for mutations at the tk locus following irradiation in L5178Y mouse lymphoma cell line (Moore et al., 1986).

Measurement of the optimum expression time

This assay was carried out for control, 2-, 4- and 6-Gy treated cells. For each dose, six 75-cm^2 flasks with densities ranging from 2×10^5 to 2×10^6 cells per flask were set up in MEM medium. Following irradiation, flasks were incubated at 37 °C for 0–5 days expression period. After the required expression period, cells were trypsinised and plated in TFT as above (mutation assay). A viability assay was carried out for each dose to determine the cell viability in the mutagenised population.

X-Ray irradiation

Cells were exposed as monolayers in medium to X-rays (250 kV with 0.5 mm Cu filtration) at a

dose rate of 0.75 Gy/min. Dosimetry was checked using a modified Fricke method (Frankenberg, 1969).

Cell survival assay (non-mutagenised population)

In order to verify the reported difference in X-ray sensitivity, a survival assay was carried out on both *xrs5* and CHO K1 cell lines. Cells were irradiated with a graded series of doses as monolayers in medium. These cells were trypsinised, appropriately diluted and plated in 5-cm dishes (Sterilin) with 5 ml MEM and incubated in a humidified incubator at 37 °C with 5% CO_2 in air for 7 days prior to fixing and staining as above.

Results

The survival curves for the two cell lines are shown in Fig. 1. The difference in the survival curve shape for the two lines confirms previous work (Jeggo and Kemp, 1983) and served as a check on the hypersensitivity of the *xrs5* line to X-irradiation in our laboratory before we initiated

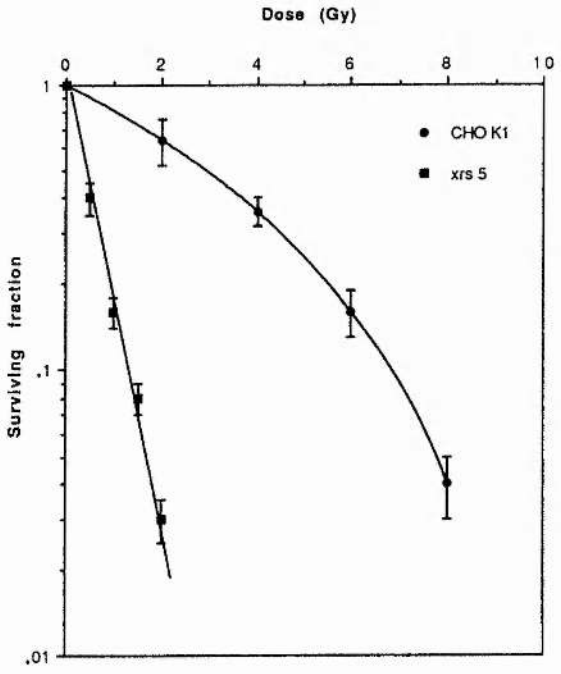

Fig. 1. Survival curves for X-irradiated CHO K1 and *xrs5* cells. Vertical bars represent standard errors of mean values. Each survival curve represents the mean of at least 3 Expts.

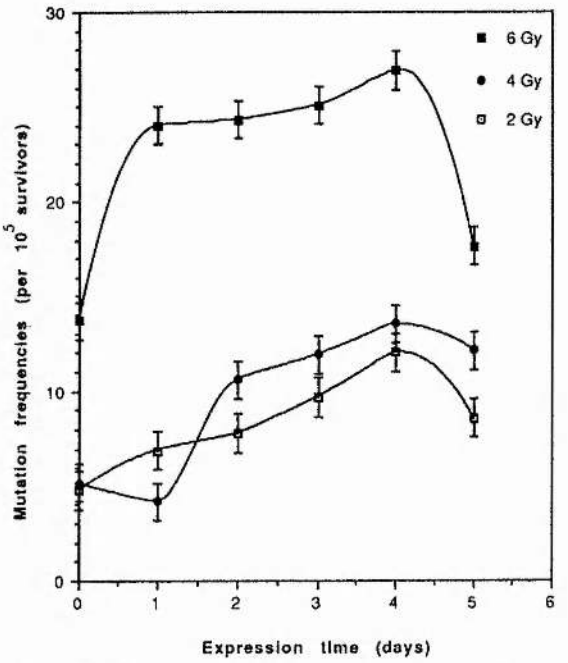

Fig. 2. Mutation frequencies as a function of post-irradiation incubation period (expression time) in the X-ray-irradiated CHO K1 cell line. Vertical bars represent standard errors of mean values. Each curve represents the mean of 3 Expts.

our mutation studies. The results of the observed mutation frequencies at various doses following different expression times are presented in Fig. 2. Expression time is defined as time between X-ray exposure and plating cells in selective medium (TFT). The results show that mutations generally increased over the first 3 days reaching a peak at 4 days followed by a decrease in mutation frequency at 5 days. A similar trend was seen in the case of the *xrs5* cell line (results not shown). Hence 4 days was taken as the optimum expression time to yield the maximum observed mutation frequency for both cell lines. The results for mutation frequency at the tk locus in the *xrs5* and the parental CHO K1 cells are shown in Fig. 3. The average spontaneous mutation frequencies of 3.64 and 10.6 per 10^5 viable cells in CHO K1 and *xrs5* respectively (Table 1) have been subtracted from the values shown. A similar 3-fold higher spontaneous mutation rate at the hprt locus in *xrs5* than in CHO K1 has been reported previously (Darroudi and Natarajan, 1989). We found that treat-

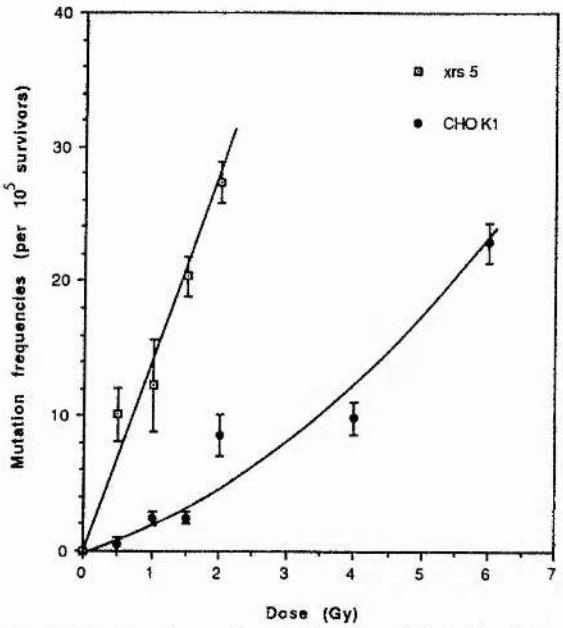

Fig. 3. Mutation frequencies as a function of dose after X-ray exposure in *xrs5* and CHO K1 cell lines. Vertical bars represent standard errors of mean values. Each curve represents the mean of 3 Expts.

ment of cells with hypoxanthine–aminopterin–thymidine (HAT) medium prior to mutagenicity experiments in order to kill any tk^- cells which had arisen spontaneously did not reduce this increased mutation rate in *xrs5*. Even after subtraction of spontaneous rates, *xrs5* showed a 3–4-fold enhanced induced mutability at the tk locus. This difference in mutation frequency is significant even in the low dose range. The data points for all the figures represent the mean of at least 3 independent experiments. The frequency of mutations in CHO K1 showed a curvilinear response with increasing X-ray dose while the *xrs5* data approximated to a linear increase in mutation frequency with increasing dose. The frequency of mutations per 10^5 viable cells was 3–4 times higher in the *xrs5* than in the CHO K1, the ratio of mutation frequency between the two cell lines varying with dose.

TABLE 1

FREQUENCY OF SPONTANEOUS TFT^R MUTANTS PER 10^5 SURVIVORS

Cell type	Spontaneous mutations (per 10^5 survivors) [a]	Standard deviation
CHO K1	3.64	2.6
xrs5	10.6	2.5

[a] Average of at least 4 independent Expts.

Discussion

Early work with *E. coli* and yeast cells suggested that repair/misrepair of various mutagenic induced damage to the DNA might be implicated in yielding the observed mutation frequency rates (Speyer, 1965; Von Borstel et al., 1968). From a study of data on radiation-sensitive mutants in yeast, Hastings et al. (1976) suggested that radiation-sensitive mutants show a different mutational response from the wild-type due to the increase in error-prone repair. Although some repair-deficient or radiation-sensitive mutants have been shown to be more mutable than their parental radio-resistant strains (Evans et al., 1986), a notable exception is A–T cells which although they are more sensitive than normal cells yet have not found to be hypermutable (Arlett, 1980; Arlett and Harcourt, 1978). From an analysis of DNA strand breaks, A–T cells have been shown to be proficient in dsb repair (Lehmann and Stevens, 1977) although at a lower fidelity rate (Cox et al., 1986). The lack of an error-prone repair process has been suggested as one of the possible factors leading to the observed hypomutability of A–T cells (Arlett and Harcourt, 1983). A recent study (Zdzienicka et al., 1988), using a V79 mutant (XR-V15B) reported that this line does not show an enhanced induced mutability despite its increased hypersensitivity to radiation and defective dsb repair. However, our results showing an enhanced mutability at the tk locus in *xrs5* when compared with the CHO K1 parent is in agreement with an earlier report (Darroudi and Natarajan, 1989) which showed that *xrs5* and *6* were hypermutable to X-rays at the hprt locus.

The frequency of mutations at the tk locus in *xrs5* and CHO K1 cells in the present study were higher than found previously for hprt mutations in Chinese hamster V79 cells (Thacker et al., 1977) and in the mouse lymphoma strain L5178Y (Evans

et al., 1986). The reason for this may be that the tk locus resides on a chromosome with non-essential flanking regions so that deletion of a large part of this chromosome does not lead to cell death. This view is supported by recent studies which suggests that the heterozygous nature of the tk locus permits the recovery of both single-gene and chromosomal mutations while the hprt locus permits the recovery of mutations affecting a single gene (Stankowski and Hsie, 1986; Evans et al., 1986; Yandell et al., 1986; Little et al., 1987; Moore et al., 1987, 1989). Trifluorothymidine-resistant clones (TFT^R) grown through many generations were found to be non-viable in HAT medium indicating their stability. With regard to the unusual findings that CHO K1 and *xrs5* are heterozygous with respect to the tk locus, we confirmed this heterozygosity in earlier work (Martin, 1986) which showed that when induced TFT^R mutants were exposed to the methylation inhibitor 5-azacytidine (Jeggo and Holliday, 1986) no revertant clones were detected when cells were plated in HAT medium. The occurrence of heterozygosity at the tk locus may not be a totally surprising result in view of the high degree of aneuploidy observed in CHO K1 cells which could have resulted from the loss of the chromosomes carrying this locus.

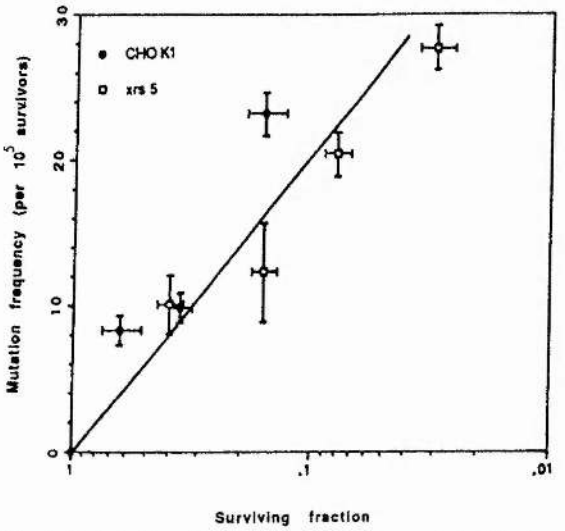

Fig. 4. A plot of mutation frequency versus surviving fraction for both CHO K1 and *xrs5* cell lines. Data represents the mean of at least 3 Expts.

Evans et al. (1986) suggested that the repair deficiency could be responsible for the occurrence of residual DNA multilocus lesions following X-irradiation in the radiosensitive strain LY-S of the mouse lymphoma cell line (L5178Y) which results in its increased mutational response. Further work with the same cell line provided a correlation between repair of dsb, chromosome damage and cellular radiosensitivity (Evans et al., 1987; Wlodek and Hittleman, 1987, 1988). In the present study, we have used the dsb repair deficient *xrs5* mutant to show a possible correlation between a deficiency in dsb repair and the enhanced mutability observed (Fig. 3). The *xrs5* cell line has been shown to have a reduced ability to repair dsb (Kemp et al., 1984; Costa and Bryant, 1988) which has been linked to its enhanced chromosomal sensitivity to X-rays (Kemp and Jeggo, 1986; Darroudi and Natarajan, 1987) and to restriction endonucleases (Bryant et al., 1987); and to their extreme cellular radiosensitivity (Jeggo and Kemp, 1983). Despite the low viability in the mutagenised population of the *xrs5* cell line, the enhanced mutation frequency suggests that the mutational events outnumber the lethal events.

A plot of mutation frequency versus survival (Fig. 4) shows that both sets of data can reasonably be fitted by a common line suggesting a common lesion type leading to expressed mutations and cell killing in both these cell lines. These results suggest the likely involvement of dsb in mutation induction as well as cell killing in irradiated mammalian cells. The low capacity of the *xrs5* mutant to repair dsb would seem likely to be the determining factor in the observed hypermutability and suggest the dsb as a pre-mutational lesion. This view is consistent with the involvement of dsb as the principal mutagenic lesion in X-ray mutagenesis (Gibbs et al., 1987). Szostak et al. (1983) suggested that dsb stimulates recombination-type repair which if unsuccessful results in substantial deletions. Therefore it seems likely that the high mutability of *xrs5* results from its inability to remove dsb from the DNA resulting in high levels of deletions.

Acknowledgements

T.M. was supported by a grant from Government of Iraq. B.S. is supported by a grant from British Nuclear Fuels plc. We wish to thank Mr. J. McIntyre for his excellent technical assistance.

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Induction of forward mutations were measured at the thymidine kinase (*tk*) locus in hamster cells.We have shown that an X-ray sensitive mutant cell line (*xrs* 5) which is deficient in dsb repair was found to be hypermutable compared to its parent (CHO KI) cell line when exposed to X-rays. This suggested that the inherently defective dsb repair system could be the causative factor and this formed strong evidence for dsb as the critical lesions in radiation-induced mutagenesis.To further investigate this, dsb repair was inhibited in CHO KI cells by exposure to X-rays in the presence of 9-ß-D-arabinofuranosyladenine (ara A). An enhanced mutational response was observed in cell treated with X-rays in combination with ara A. This suggests that unrepaired dsb may be important lesions in the steps leading to mutations. In further experiments, CHO KI cells were treated with Restriction endonucleases, either Pvu II or Eco RI both of which generate blunt and cohesive-ended dsb respectively. Results showed higher (10 fold) mutation frequencies in Pvu II treated cells compared to cells treated with equal concentrations of Eco RI. These results indicate that blunt-ended dsb are more effective than cohesive-ended dsb in mutation induction. This may result from the slower repair of blunt-ended dsb than cohesive-ended dsb. In conclusion, we suggest that dsb represent pre-mutational lesions which are expressed as mutations possibly via the formation of deletion or exchange type of chromosomal aberrations.